How Does Synaptotagmin Trigger Neurotransmitter Release?

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Key Words
fusion pore, membrane fusion, SNARE, synapse

Abstract
Neurotransmitter release at synapses involves a highly specialized form of membrane fusion that is triggered by Ca^{2+} ions and is optimized for speed. These observations were established decades ago, but only recently have the molecular mechanisms that underlie this process begun to come into view. Here, we summarize findings obtained from genetically modified neurons and neuroendocrine cells, as well as from reconstituted systems, which are beginning to reveal the molecular mechanism by which Ca^{2+}—acting on the synaptic vesicle (SV) protein synaptotagmin I (syt)—triggers rapid exocytosis. This work sheds light not only on presynaptic aspects of synaptic transmission, but also on the fundamental problem of membrane fusion, which has remained a puzzle that has yet to be solved in any biological system.
INTRODUCTION

Presynaptic nerve terminals perform the highly specialized task of transmitting chemical signals to target cells with exceptionally high spatial and temporal precision. Transmission is mediated by the Ca\textsuperscript{2+}-triggered fusion of transmitter-filled synaptic vesicles (SVs) with the presynaptic plasma membrane. This is the fastest known membrane fusion event in cells, occurring in milliseconds, suggesting that the mechanism of fusion involves a limited number of protein and lipid rearrangements (1–3). The goal of this review is to present current thinking about what occurs during this window of time.

As a starting point, we briefly review the SV cycle with emphasis on the Ca\textsuperscript{2+}-activated fusion step. We then describe the structure of synaptotagmin I (syt), a Ca\textsuperscript{2+}-binding protein proposed to regulate the fusion step. (Hereafter, synaptotagmin-I is referred to as syt except where different isoforms are compared; in those cases, syt will be followed by a Roman numeral.) This is followed by a discussion of genetic studies aimed at determining whether syt regulates exocytosis at synapses, leading to kinetic experiments (using neuroendocrine cells) that are directed toward understanding the function of syt during membrane fusion. Finally, we narrow our discussion to address the ability of syt to regulate membrane fusion as a consequence of its ability to act on its effectors in reconstituted systems. These effectors include anionic phospholipids, e.g., phosphatidylserine (PS) (4), as well as soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs).
(SNAREs) that form the core of a conserved membrane fusion machine at synapses (5).

OVERVIEW OF THE SYNAPTIC VESICLE CYCLE: DISTINCT MODES OF EXOCYTOSIS

Synaptic vesicles recycle locally within presynaptic boutons. This cycle has been discussed in detail in a number of excellent reviews (6–8) and is recapitulated in Figure 1. Briefly, proton pumps acidify the lumen of SVs, and the resultant pH gradient drives transporter-mediated uptake of neurotransmitters (9, 10). Loaded SVs are targeted to specialized sites of release, termed active zones (11). After docking/attachment to these release sites, vesicles undergo priming steps that render them competent to fuse (12, 13); the docking and priming steps are reversible (14, 15). Upon arrival of an action potential, exocytosis is triggered by influx of Ca\(^{2+}\) ions through voltage-activated Ca\(^{2+}\) channels (1). After fusion, the vesicle is retrieved by endocytosis and reloaded for another round of exocytosis (6, 16). Interestingly, newly retrieved vesicles might not correspond to those that just underwent exocytosis (17, 18).

The question of whether SVs always fuse with the plasma membrane, or can release at least some of their contents through the reversible opening of a fusion pore without membrane merger (Figure 1), remains the subject of debate (reviewed in References 19 and 20; also see References 21 and 22). It is important to note that both modes of exocytosis have been clearly established for large dense-core vesicle (LDCV) exocytosis in neuroendocrine cells. For example, Fulop et al. (23) showed that strong stimuli in chromaffin cells result in complete fusion and lipid mixing but that fusion pore expansion and lipid mixing do not occur when secretion is driven by weaker stimuli (23, see also 24–26).

Owing to their small size, and other technical considerations, it is more difficult to distinguish distinct modes of SV exocytosis. However, two modes of styryl dye release from single SVs in mouse hippocampal neurons have been reported (27). Some SVs appear to lose all of the dye abruptly and completely, whereas others slowly release only

Figure 1
The synaptic vesicle cycle. Please see text for details. Abbreviation: NT, neurotransmitter.
Kiss and run: a form of exocytosis in which the transmitter can be expelled without complete fusion and collapse of secretory vesicles

SNARE complex: a hetero-trimeric protein complex formed by interactions between vesicle proteins (e.g., synaptobrevin) and target membrane proteins (e.g., syntaxin and SNAP-25)

v-SNARE: vesicle SNARE
t-SNARE: target membrane SNARE

C2 domain: a widely occurring conserved motif of \( \sim 140 \) amino acid residues, which often binds Ca\(^{2+} \) and membranes

a fraction of the dye (28). This latter mode of release is consistent with kiss-and-run exocytosis through nondilating fusion pores. Capacitance measurements of SV exocytosis in the calyx of Held also support the notion that kiss-and-run exocytosis occurs and might give rise to distinct physiological responses as compared to full fusion (29). In addition, capacitance measurements of small synaptic-like microvesicle exocytosis in the posterior pituitary revealed kiss-and-run fusion events with fusion pores that were only \( \sim 0.6 \) nm in diameter (30). Such tiny pores might go undetected by current methods used to study SV exocytosis. For example, 0.6-nm pores are unlikely to allow efflux of styryl dyes such as FM-43 (28), and if they occur at central synapses, they would be expected to either fail to give rise to an AMPA receptor response (31, 32) or would show attenuated postsynaptic responses (29, 33, 34). Finally, transmitter release through tiny fusion pores might desensitize, rather than activate, postsynaptic receptors (28, 30, 35).

It is intriguing to consider the existence of varying sizes of fusion pores, some of which would escape detection using standard optical and electrophysiological measurements. If presynaptic nerve terminals employ fusion pores with different properties, switching between these modes could underlie aspects of synaptic plasticity (36). This notion underscores the need for new ways to study SV exocytosis and raises the point that much remains to be discovered regarding presynaptic aspects of synaptic transmission.

SNARES ASSEMBLE INTO A MEMBRANE FUSION MACHINE

The core of the membrane fusion machinery is formed by a conserved set of vesicle and target membrane proteins (37–39)—called SNAREs—that assemble together into highly stable complexes. The SNARE complex was discovered by Rothman and colleagues (40) and has been studied and discussed in considerable detail; we refer the reader to a number of recent comprehensive reviews [e.g., (12, 13, 41, 42)]. Briefly, neuronal exocytosis is mediated by the vesicular (v-) SNARE synaptobrevin/VAMP and the target membrane (t-) SNAREs syntaxin and SNAP-25. The core of this complex is a four-helix bundle in which SNAP-25 contributes two helices, and synaptobrevin and syntaxin—with their membrane anchors embedded in the vesicle and plasma membrane, respectively—contribute one helix each (43, 44). v- and t-SNAREs assemble into trans-complexes, and these appear to be necessary and sufficient for fusion (Figure 2) (5, 41). The mechanism by which SNAREs catalyze fusion and how this process is regulated by Ca\(^{2+} \) at synapses are major questions for which we are only now beginning to find answers (Figure 2).

DISCOVERY AND STRUCTURE OF SYT: A Ca\(^{2+} \) SENSOR FOR EXOCYTOSIS

The classical work of Katz and colleagues established that SV exocytosis is triggered by Ca\(^{2+} \) (1, 45). Identification of the Ca\(^{2+} \) sensor that regulates the fusion reaction would open the door to tracing this transduction pathway, thus providing a means of elucidating the mechanism of regulated exocytosis. A breakthrough came in 1981, when Matthew et al. (46) identified a 65-kDa protein, syt, which was localized to SV and LDCVs in neurons and neuroendocrine cells. The primary sequence of syt revealed a single membrane-spanning domain, a short intraluminal domain, and a large cytoplasmic domain consisting of tandem C2 domains, C2A and C2B, connected by a linker (Figure 3a) (47, 48).

C2 domains are found in a variety of proteins where they often serve as Ca\(^{2+} \)- and phospholipid-binding modules (49); in syt, both C2 domains “sense” Ca\(^{2+} \) (4, 50–52). In addition to syt-I, 16 additional isoforms have been identified (52a; see also the HUGO gene nomenclature committee at http://www.genenames.org). This review
SNARE function during the late steps of membrane fusion. (a) The mechanisms that underlie docking of synaptic vesicles (SVs) and large dense-core vesicles (LDCVs) are not well understood, so the proteins that mediate docking are depicted with a (?); however, recent studies suggest that docking involves Munc18/nSec1/ribSec1 and UNC-10/Rim (197, 198). It should also be noted that the mechanisms of SV and of LDCV docking might differ, and the roles of SNARE proteins, which were originally thought to not play roles in docking of either sort of organelle (e.g., (199, 200)), are reemerging as key players in this process, at least in the case of syntaxin (201, 202). After docking, vesicles undergo priming but are quiescent until arrival of the Ca\(^{2+}\) signal. At this step, it is not yet known whether trans-SNARE pairing is blocked or whether SNARE complexes are partially or fully assembled, so all three possibilities are drawn in the docked but quiescent state. Upon arrival of the Ca\(^{2+}\) signal, partially assembled or unassembled SNAREs further assemble (203), or fully assembled SNARE complexes undergo undefined rearrangements. This step is associated with the opening of the fusion pore. If kiss and run occurs, the fusion pore closes without dilating; if full fusion occurs, the pore expands, and the vesicle collapses into the plasma membrane (detailed in Figure 4). The composition and structure of the fusion pore have not been definitively determined, but two studies indicate that in its initial open state, the fusion pore that mediates transmitter release from LDCVs in PC12 cells is lined with the membrane anchor of the t-SNARE syntaxin (194, 195). It has also been suggested that the fusion pore is purely lipidic (159), or lined with another protein (shown in yellow) that interacts with SNARE proteins (204); all three possibilities are shown, and these models are not mutually exclusive (42). (b) A hemifusion intermediate, in which the proximal leaflets merge before the distal leaflets, is consistent with either protein-lined or lipidic fusion pores. The models outlined in panels (a) and (b) are discussed in greater detail in a recent review on fusion pores (42).
Figure 3
Structure of synaptotagmin I (syt) and the phenotype of syt null neurons. (a) Domain structure of syt; the C2A and C2B domains were rendered from References 205 and 58, using PyMOL (DeLano Scientific LLC). The remaining protein segments were added using a drawing program. Ca2+ ions are depicted as spheres. The side chains of R233, K366, Y311, K326, and K327 are shown. Abbreviation: TMD, transmembrane domain. (b) Excitatory postsynaptic currents from wild-type (WT) and syt-/- autaptic cultures of hippocampal neurons; synchronous transmission is reduced, with a concomitant increase in asynchronous release. (c) Cumulative charge from recordings like those shown in panel (a); synaptic transmission is markedly slower in syt-/- autapses. Abbreviation: KO, knockout. (d) Total charge transfer was similar in WT and syt null neurons. Panels b-d are reprinted with permission (53).

is largely focused on the first isoform, syt-I, which appears to play a critical role in exocytosis in a variety of inhibitory as well as excitatory neurons (53, 54).

The crystal structure of the C2A domain of syt-I, and the cytoplasmic domain (designated C2A-C2B) of syt-III, revealed that C2 domains are compact eight-stranded β-barrels, with two protruding loops that form the Ca2+-binding pockets (Figure 3a) (55, 56). Nuclear magnetic resonance (NMR) studies indicate that five acidic amino acid side chains mediate binding of three Ca2+ ions in C2A (via D172, D178, D230, D232, and D238) and ≈2 Ca2+ ions in C2B (via D303, D309, D363, D365, D371) (Figure 3a) (57, 58). However, a more recent structural study indicates that D371 does not serve as a Ca2+ ligand in C2B (59).

The localization of syt to SVs, coupled with its ability to bind Ca2+, prompted the idea that this protein might function as a Ca2+ sensor that regulates exocytosis (4). Before delving into this latter point, it should be noted that syt is a multifunctional protein that appears to be involved in numerous aspects of
the SV cycle, including docking in *Drosophila* (60) and endocytosis in *Caenorhabditis elegans* (61), *Drosophila* (62, 63), and mouse neurons (64). It has been reported that there is no qualitative difference in overall bouton morphology between wild-type (WT) and syt knockout (KO) mice (65), but whether syt plays a role in SV docking awaits quantitative ultrastructural analysis of syt KO neurons. In the following sections, we restrict our discussion to the function of syt during Ca\(^{2+}\)-triggered exocytosis.

**GENETIC STUDIES OF SYT**

**Reevaluation of the Syt Null Phenotype**

Disruption of the syt gene in a variety of organisms demonstrated that this protein plays a critical role in synaptic transmission (66). Although syt-deficient worms, flies, and mice have been analyzed over the last 14–15 years, recent studies are reshaping our view as to the precise physiological function of this protein, and a number of aspects of the syt null phenotype are currently in flux. In the following section, we summarize new findings that have altered our view of how syt might function at synapses.

**Impact of Syt on the Kinetics of Neurotransmitter Release: An Accelerator of Exocytosis?**

There are two competing views as to the role of syt in SV exocytosis. In early studies, which made use of paired recordings from small numbers of excitatory hippocampal neurons grown on “microislands,” it was concluded that disruption of syt largely abolished the rapid synchronous component of exocytosis; the slower asynchronous phase of release, which occurs over the next few hundred milliseconds following the rapid synchronous phase, was unaffected (65). In follow-up studies, based on paired recordings from mixed cultures of cortical neurons, this laboratory further confirmed that asynchronous release is not significantly altered in syt null synapses (7, 54). Because >90% of the total release (or charge transfer) is mediated by the synchronous component, and because asynchronous release was reported to be unaffected in the knockouts, these studies indicated that syt plays an essential role in mediating the total amount/extent of exocytosis.

In parallel with these studies in mice, Yoshihara & Littleton (67), working in flies, proposed a new model in which syt is not needed to enable SV exocytosis per se but rather functions to synchronize exocytosis during stimulation, such that release occurs very rapidly in response to depolarization. They discovered that, at the neuromuscular junction (NMJ) of *Drosophila* larvae lacking syt, significant levels of evoked release still occurred, but this release was no longer tightly coupled, in time, with depolarization. These findings suggested that syt is needed to convert the asynchronous release events into rapidly triggered synchronous events.

More recently, quantitative studies by Nishiki & Augustine (53, 68), who recorded from autaptic cultures of hippocampal neurons, demonstrated that the total charge transfer (i.e., amount of exocytosis) in WT and syt -/- autapses were the same, indicating that syt is not needed for normal levels of release (**Figure 3d**). However, in the nulls, the rate of release was dramatically reduced, and virtually all exocytosis became asynchronous (**Figure 3b, c**). So, the newly emerging view, from both the *Drosophila* NMJ and autaptic cultures of mouse hippocampal neurons, is that syt functions to synchronize and accelerate Ca\(^{2+}\)-triggered membrane fusion. These findings are also consistent with the idea put forward by Neher and colleagues (69) that an alternative function for syt could be in the formation, or stabilization, of a pool of vesicles capable of being rapidly released.

The idea that syt is not essential for normal levels of SV exocytosis represents somewhat of a paradigm shift in our understanding of syt function. These new findings prompt
numerous questions, including the identification of the Ca$^{2+}$ sensor that mediates efficient, albeit slow, exocytosis in the absence of syt-I. Importantly, why different groups perform similar experiments and arrive at such different conclusions remains unresolved. It has been argued that the conflicting results can be explained by the idea that asynchronous release is elevated only in autaptic cultures but not in pairs of connected neurons in dissociated cultures (54). However, in dissociated cultures from syt knockouts, the SV cycle remains functional; normal levels of FM dye are taken up and released (53, 64). Furthermore, asynchronous release is elevated in the syt null fly NMJ (67).

**Spontaneous Release Events: Is Syt a Fusion Clamp?**

The studies outlined above demonstrate that syt plays a positive role in secretion, but syt might have a dual function in the regulation of release. In particular, an early model postulated that syt serves as a clamp that blocks SNARE-catalyzed fusion until the arrival of a Ca$^{2+}$ signal (70, 71). If this idea is correct, disruption of syt might be expected to result in higher rates of unregulated, or spontaneous, SV exocytosis at synapses. The clamp model remains in flux, as detailed below.

A clamping function was supported by early studies of synaptic transmission at the NMJ of syt-/- *Drosophila* larvae, where increases in the rate of spontaneous SV fusion events (called minis) were independently reported by multiple groups (72–74). Similar studies were carried out using the more difficult *Drosophila* embryo preparation, and mixed results were obtained, ranging from an increase (75) to no change (67, 76) in mini frequency. Acute inactivation of syt at the larval NMJ, using chromophore-assisted light inactivation, revealed a short-lived increase in mini frequency, which almost immediately (within minutes) decayed back to WT levels of spontaneous release (although C2B, and probably not C2A, was disrupted) (77). Finally, there was no change in mini-frequency in cultured hippocampal neurons (grown on microislands) from syt knockout mice (65). In contrast, elevated mini frequencies in dissociated mouse cortical neurons lacking syt were recently reported (78). Thus, there remains some uncertainty as to whether syt plays a role in preventing fusion until the arrival of the Ca$^{2+}$ signal, but the majority of reports indicate that syt does act to prevent spontaneous fusion and hence appears to have some “clamp-like” function in nerve terminals.

**Ca$^{2+}$ Ligand Mutations in Syt: Impact on Synaptic Transmission**

The Ca$^{2+}$-binding sites of syt can be disrupted by point mutations that neutralize the acidic Ca$^{2+}$ ligands in each C2 domain. Expression of these mutants in syt null neurons provides a means to address the function of these Ca$^{2+}$-binding sites in synaptic transmission. If syt is a major Ca$^{2+}$ sensor for exocytosis, one might expect that loss of Ca$^{2+}$-binding activity would reduce the Hill coefficient for the Ca$^{2+}$ dependence of release from ~4 to a smaller value (79, 80). Surprisingly, a number of twists have arisen from these mutagenesis studies. For example, when all five Ca$^{2+}$ ligands in C2A were neutralized, such that C2A fails to bind Ca$^{2+}$ altogether, there was no apparent defect in synaptic transmission (81; see also 82). In contrast, neutralization of a single specific Ca$^{2+}$ ligand (D232N) enhanced release in a manner consistent with the activation of a Ca$^{2+}$-binding site (81, 83; see 84 for a different view). It is a rather remarkable observation that the quintuple mutation failed to disrupt synaptic transmission (81). Yet, all of the Ca$^{2+}$ ligands in C2A are conserved throughout evolution and are likely to subserve some role that has eluded detection in these experiments.

Substitution of the Ca$^{2+}$ ligands in C2B resulted in a somewhat clearer picture; some of
the Ca\textsuperscript{2+} ligands in C2B are absolutely essential for syt function (68, 74). However, it appears that individual Ca\textsuperscript{2+} ligands within C2B play different roles: Substitution of one putative Ca\textsuperscript{2+} ligand (D371N) (see Reference 58 versus 59) had no discernible effect, whereas substitution of other Ca\textsuperscript{2+} ligands (D309N or D363N) completely disrupted function (68, 74). In addition, when expressed in syt -/- hippocampal autapses, different Ca\textsuperscript{2+} ligand mutants produced a graded suppression of the elevated asynchronous release that results from disruption of syt (53, 68).

Why does neutralization of different Ca\textsuperscript{2+} ligands in an individual C2 domain, C2A or C2B, have different consequences (68, 74, 81, 82, 85)? At a superficial level, the PS- and SNARE-binding activities of syt (discussed in further detail below) appear to be affected in a similar manner by disruption of pairs of Ca\textsuperscript{2+} ligands in the C2A and C2B domains (e.g., Reference 86), yet Ca\textsuperscript{2+} binding to C2B plays a much more critical role in synaptic transmission. Two possible explanations come to mind: C2B engages PS and SNARE proteins in a manner that is somewhat distinct from the adjacent C2A domain (87–90), and C2B selectively participates in additional interactions, which might also be critical for fusion [e.g., binding to phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) (91, 92) and self-association (93)].

Detailed quantitative analysis is needed to more precisely discern the biochemical consequences that result from neutralizing Ca\textsuperscript{2+} ligands in the C2 domains of syt. These experiments will include time-resolved measurements of the ability of syt to penetrate and tubulate membranes (90, 92), to drive folding and assembly of SNARE proteins in vitro (94), and to regulate the dynamics of fusion pores (95, 96), as discussed in detail below.

**Other Mutations that Affect the Ca\textsuperscript{2+}-Sensing Ability of Syt**

The first studies to show that mutations in syt can affect the Ca\textsuperscript{2+} requirements for exocytosis were carried out at the *Drosophila* NMJ. These studies revealed point mutations in syt that altered the Ca\textsuperscript{2+} requirements for exocytosis and indicated that syt functions as part of an oligomeric complex (72, 73). For example, the AD3 mutant allele harbors a point mutation (shown in Figure 3a as Y311N in the mouse sequence) that disrupts the Ca\textsuperscript{2+}-sensing ability of C2B, shifts the Ca\textsuperscript{2+} requirements for exocytosis to the right, and impairs exocytosis at a step after SV vesicle docking (62, 72). These findings indicate that the C2B domain of syt must sense Ca\textsuperscript{2+} in order for docked SVs to fuse in response to Ca\textsuperscript{2+}.

Similar studies have been carried out in mice. Namely, substitution of a charged residue in the C2A domain, R233Q, which plays a role in both syt-membrane and syt-SNARE interactions (Figure 3a) (discussed below and in Reference 97), altered the Ca\textsuperscript{2+} requirements for SV exocytosis in cultured hippocampal neurons (98). At the biochemical level, this is a rather subtle mutation, as it shifts the Ca\textsuperscript{2+} requirements for C2A-membrane interactions to the right by a factor of two (97, 98). In contrast, as detailed above, neutralization of a Ca\textsuperscript{2+} ligand in the same C2 domain, C2A (D232N), abolishes the interaction of the C2A domain with phospholipids (99, 100) but either has no effect (84) or results in enhanced exocytosis (81, 83). Why does a partial loss-of-function mutation in C2A (i.e., R233Q) have a stronger affect on synaptic transmission than Ca\textsuperscript{2+} ligand mutations that abolish C2A-membrane interactions? Analysis of the R233Q mutation in the context of the intact cytoplasmic domain of syt revealed that this mutation diminishes the ability of the adjacent C2B domain to bind anionic lipids as a function of [Ca\textsuperscript{2+}] (97, 101). In contrast, complete disruption of C2A-membrane interactions (99, 100) has no apparent effect on the Ca\textsuperscript{2+} dependence for syt-membrane interactions (84) mediated by the intact, adjacent C2B domain (101). So, an intriguing possibility is that the R233Q mutation somehow affects the cooperative interaction between C2A and C2B (101, 102),
such that C2B is rendered less able to trigger release.

In summary, data garnered from mutant forms of syt are consistent with the idea that syt functions as a Ca\(^{2+}\) sensor that regulates SV exocytosis. However, it should be emphasized that the changes in synaptic transmission that result from loss-of-function mutations in syt are mimicked by mutations in a number of other proteins, many of which do not bind Ca\(^{2+}\) (66), so these experiments do not provide definitive proof that syt is a Ca\(^{2+}\) sensor for rapid secretion. Further insight into the function of syt could be gained by experiments that address the action of syt during membrane fusion. In the next section, we describe another kind of experiment, which suggests that, upon binding Ca\(^{2+}\), syt regulates the final steps of LDCV exocytosis—the opening and dilation of fusion pores.

**SYT REGULATES FUSION PORE DYNAMICS**

Another approach to study the function of proteins during membrane fusion is carbon fiber amperometry, which directly monitors individual fusion events. The benefit of this approach is that transmitter flux can be detected in real time, revealing intermediates in the fusion reaction. A drawback of this method is that it is limited to the detection of readily oxidized molecules (e.g., dopamine, norepinephrine, and serotonin) and cannot currently be used to study release of the major transmitters that mediate fast synaptic transmission between neurons (e.g., glutamate and GABA). So, this method has been largely applied to the study of catecholamines from LDCVs in neuroendocrine cells.

By carrying out amperometric recordings of LDCV exocytosis from chromaffin cells, Chow et al. (103) observed that, in its initial open state, the fusion pore, which corresponds to the first aqueous connection between the vesicle lumen and the extracellular fluid, often gave rise to a tiny trickle of transmitter before it subsequently dilated. This is illustrated in Figure 4 (upper panels), where amperometric traces of two kinds of release events are shown. The fusion pore opens quickly, and its open lifetime distribution is well fitted by a single exponential function. The open fusion pore has two choices, it can close (resulting in kiss-and-run exocytosis), or it can dilate, resulting in full fusion (Figure 4). Because of the well-behaved nature of this lifetime distribution \[\tau = 1/(k_c + k_d);\] rate constants are defined in Figure 4, quantitative measurements of fusion pore stability are possible (95, 104).

Recent studies revealed that the rate of LDCV fusion pore dilation is regulated by the intracellular Ca\(^{2+}\) concentration (26, 105), which is consistent with the idea that syts regulate the final stages of membrane fusion. Direct support for this model came from experiments in which the ratio of different syt isoforms, on LDCVs in PC12 cells, was altered (95). These organelles harbor syt-I and syt-IV (95, 106–108), as well as syt-VII (109, 110) and syt-IX (111, 112). Syt-IV is unusual in that it fails to bind PS in response to Ca\(^{2+}\) (113), and its expression is rapidly induced by activity (106, 114–118). Upregulation of syt-IV reduced the mean open lifetime of fusion pores such that pore closure was favored over pore dilation (i.e., syt-IV destabilizes the pore) (95). Upregulation of syt-IV also increased the frequency of long-lived \((\sim 80\) ms) tiny pores (that have one fifth the flux of a prespike foot) that always closed without dilating (107). In contrast, overexpression of syt-I increased the open lifetime of prespike feet, indicating that this isoform stabilizes fusion pores (95). It has been suggested that syts might alter fusion pore properties through a nonspecific mechanism by altering membrane composition or tension (7), but this concern can be ruled out by the observation that upregulation of syt-I and syt-IV have opposite effects on fusion pore open lifetime distributions (95).

Using overexpressed WT syt as a reference point, mutant syts were expressed and analyzed in PC12 cells. One set of
Figure 4

The C2B domain of syt controls fusion pore dilation in PC12 cells. The illustration in the center shows a fusion pore that initially opens to small diameter; this gives rise to a small flux of transmitter (transition from the closed to the open state). This is detected by carbon fiber amperometry as a prespike foot (PSF) (upper panels); this is the initial open state of the fusion pore. The fusion pore can either close (upper left), yielding kiss and run (upper right), or it can dilate (transition from the open state to the dilated state), resulting in an amperometric spike (upper right). Overexpression of a mutant form of syt, wherein Ca\(^{2+}\) binding to the C2B domain was disrupted by a point mutation (D363N; syt-I-C2B\(^*\)), resulted in a greater fraction (X) of kiss-and-run events; these are called “kiss-and-run large” (KRL) to distinguish them from another class of kiss-and-run events, characterized in Reference 107, which have one fifth the amplitude of the prespike foot signals discussed here. Overexpression of wild-type syt (syt-I) had little effect on the fraction of kiss-and-run events, whereas overexpression of a Ca\(^{2+}\) ligand mutant in the C2A domain (D230S; syt-I-C2A\(^*\)) resulted in a slightly reduced fraction of kiss-and-run events (plotted in the lower left graph). These experiments indicate that dilation of fusion pores requires Ca\(^{2+}\) binding to the C2B domain of syt (lower right inset), providing evidence for the idea that Ca\(^{2+}\)-syt regulates exocytosis during the fusion step (95, 96). Data in the lower left graph are from Reference 26.
mutants—with graded reductions in t-SNARE-binding activity (syt-t-SNARE interactions are discussed in detail below)—gave rise to graded reductions in (a) the overall rate of secretion and (b) the open lifetime of fusion pores (96). These data strongly indicate that fusion pores are controlled, at least in part, by Ca\(^{2+}\)-regulated interactions between syt and SNARE proteins. Another mutant, in which Ca\(^{2+}\) binding to the C2B domain of syt was disrupted by substitution of a critical Ca\(^{2+}\)-binding residue (D363N), resulted in fusion pores that open but are more likely to close than dilate (Figure 4) (26). Thus, Ca\(^{2+}\) binding to the C2B domain of syt appears to play a pivotal role in fusion pore dilation. In contrast, the R233Q mutation, introduced either by overexpression in PC12 cells or by a knockin approach in chromaffin cells, reduced the rate of exocytosis but had no effect on fusion pore kinetics (97, 119). This is the only case, thus far, where overexpression and knockin methods were directly compared in terms of their impact on fusion pores, but the agreement regarding the R233Q data helps alleviate concerns inherent in each of these approaches.

Different isoforms of the v-SNARE synaptobrevin, when expressed on LDCVs in chromaffin cells, also give rise to fusion pores with distinct mean open lifetimes (104). In parallel, it was found that overexpression of the inhibitory SNARE-like protein, amisyn (120, 121), can alter the open lifetime distribution of prespike feet (122). These data are particularly interesting in light of the observation that decreases in the expression levels of SNARE proteins diminish the Ca\(^{2+}\)-cooperativity for exocytosis in Drosophila (123). A way to consolidate all of these observations is the idea that a complex of lipids, Ca\(^{2+}\), syt, and SNAREs form the core of the regulated fusion machine (Figure 5a), such that changes in any one of these components affect the function of the entire complex (94, 124, 125). In the following section, we discuss this complex in greater detail.

MOLECULAR MECHANISM OF SYT FUNCTION: INSIGHTS FROM RECONSTITUTED SYSTEMS

Syt interacts with a number of putative effectors (reviewed in detail in Reference 126). We focus on the ability of syt to bind membranes and SNARE proteins because significant progress has been made in establishing these interactions as coupling steps in regulated membrane fusion. It should also be noted that a number of syt isoforms homom and hetero-oligomerize with one another (4, 127). In some cases, oligomerization was promoted by Ca\(^{2+}\), raising the possibility that these interactions might regulate exo- and/or endocytosis (e.g., 52, 128–132). This idea was called into question by reports demonstrating that purified, recombinant syt is unable to self-associate in response to Ca\(^{2+}\) after removal of a nucleic acid contaminant (133, 134). However, a number of syt isoforms, immunoprecipitated from detergent extracts of purified SVs or transfected cells, exhibit Ca\(^{2+}\)-triggered oligomerization activity (135, 136). So, multimerization appears to be a bona fide property of syts, but may require cofactors. Detailed analysis of oligomerization has been hampered by a lack of quantitative assays that can be used to study this interaction using purified components.

Ca\(^{2+}\)-Triggered Membrane-Penetration Activity of Syt

Ca\(^{2+}\)-syt can bind (4) and aggregate (Figure 6a) vesicles that harbor anionic phospholipids (e.g., PS) (130, 137, 138). Through the use of site-directed fluorescent probes, it was discovered that in response to Ca\(^{2+}\) the Ca\(^{2+}\)-binding loops of C2A partially insert into PS/phosphatidylcholine (PC) bilayers where the probes encounter membrane-embedded fluorescence quenchers (100, 124, 139). It was originally thought that C2B did not bind PS-harboring membranes in response to Ca\(^{2+}\), but this was due to a point
mutation in the original syt cDNA (47), which disrupted the Ca\textsuperscript{2+}-sensing ability of C2B (52). It is now known that isolated C2B binds PS/PC membranes, but this interaction is weaker than isolated C2A (58, 134) and is extremely sensitive to ionic strength (140). In contrast, isolated C2B avidly binds to membranes that harbor PIP\textsubscript{2} (91, 92).

A Ca\textsuperscript{2+}-independent mode of binding to PIP\textsubscript{2} might serve to “steer” the membrane-penetration activity of syt toward the target (i.e., plasma) membrane (Figure 5a) (92). Although it has been argued that the levels of PIP\textsubscript{2} are too low in cells to mediate steering (141), PIP\textsubscript{2} is highly concentrated in microdomains where it colocalizes with syntaxin, and these domains occur at sites where LDCV exocytosis occurs in PC12 cells (142). Thus, local PIP\textsubscript{2} concentrations

**Figure 5**
The tandem C2 domains of syt simultaneously penetrate membranes to regulate reconstituted, SNARE-mediated membrane fusion. (a) Model of the Ca\textsuperscript{2+}-syt-membrane-SNARE complex (89, 94, 124). (b) The distal tips of the Ca\textsuperscript{2+}-binding loops in the C2A and C2B domains of syt rapidly penetrate into phosphatidylserine (PS)/phosphatidylcholine (PC) bilayers. The measurements used to generate this scheme are from References 92, 100, 101, 124, 140, 206. Ca\textsuperscript{2+} ions are shown as red spheres. (c) Schematic depicting an in vitro fusion assay used to monitor fusion between v-SNARE liposomes (V) (which harbor a fluorescence donor (yellow) and acceptor (red)) at sufficient levels to give rise to fluorescence resonance energy transfer (FRET) and t-SNARE liposomes (T). Upon fusion, the fluorescence (h\textnu) of the donor increases owing to loss of FRET (5, 125). (d) In vitro fusion data using liposomes that do (+PS) and do not (−PS) harbor PS (152). Absence of PS has no effect on SNARE-mediated fusion but completely abolishes the ability of Ca\textsuperscript{2+} and the cytoplasmic domain of syt to stimulate fusion. Moreover, Ca\textsuperscript{2+}-syt inhibits fusion when PS is omitted, presumably by binding to t-SNAREs in a manner that prevents assembly into viable trans-SNARE complexes. Panel a is adapted with permission from Reference 207; panel d is an alternative representative example of an experiment reprinted with permission from Reference 152.
Figure 6

Ca²⁺·syt aggregates and tubulates liposomes. (a) Tomographic reconstruction of vesicle clusters induced by Ca²⁺ and the cytoplasmic domain of syt. Arrows indicate regions of mass density where the vesicles come into contact with each other. (b) Negative stain electron micrographs of liposomes that form tubules upon incubation with Ca²⁺ and the cytoplasmic domain of syt. Images are reprinted with permission from Reference 138; see also References 90, 134, and 156.

are probably relatively high at release sites. Although PIP₂ plays an essential role in LDCV exocytosis (143, 144), it remains an open question as to whether this lipid plays an important role in SV fusion.

A surprising finding was that the PS-binding activity of C2B is markedly enhanced by the presence of an adjacent C2A domain (101, 140), suggesting that C2A and C2B cooperate to bind to membranes. One idea is that each C2 domain influences the structure of the bilayer to change the lipid environment experienced by the adjacent C2 domain (90, 145). This model might account for the ability of the R233Q mutant (described above) to affect the ability of the adjacent C2B domain to bind PS as a function of [Ca²⁺] (97, 98). However, this model for cooperativity does not explain the observation that C2A, even when mutated such that it does not bind Ca²⁺ or membranes, can still enhance the membrane-binding activity of the adjacent C2B domain (101, 140). Because the tandem C2 domains of syt appear to function in a cooperative manner, biochemical analysis of syt has shifted toward the study of the full cytoplasmic domain, rather than isolated C2 domains.

In summary, it is now firmly established that both Ca²⁺-binding loops of C2A and C2B, within the intact cytoplasmic domain of syt, simultaneously penetrate membranes that harbor PS (Figure 5a, b) (92, 102, 140). These interactions occur with diffusion-limited kinetics and generate high-affinity protein-membrane complexes (92, 100, 101, 124, 140). The Ca²⁺-binding loops of C2 domains from other proteins also interact directly with membranes (49, 146, 147), indicating that this is a general mechanism by which C2 domains engage lipid bilayers.

Mutations that Affect the Membrane-Penetration Activity of Syt

Before delving further into in vitro studies of syt·PS interactions, we first describe recent efforts to address the function of the membrane-penetration activity of syt during synaptic transmission. Rosenmund and colleagues (148) studied the effects of Trp residues placed in the distal tips of the membrane penetration loops of C2A and C2B (Figure 3a and Figure 5b). These mutations decreased the Ca²⁺ requirements for sedimentation of the cytoplasmic domain of syt.
with liposomes that harbored anionic phospholipids (148). Presumably, this effect was due to an increase in the affinity of syt for membranes that, via free energy coupling (149), increased the apparent affinity of the syt-membrane complex for Ca\(^{2+}\). When expressed in neurons, these mutants shifted the Ca\(^{2+}\) requirements for exocytosis to the left (148). A key observation was that Trp residues, placed in the loops of either C2A or C2B, enhanced exocytosis (albeit somewhat asymmetrically), supporting the idea that both C2 domains serve as functional Ca\(^{2+}\) sensors in neurons (81, 148). These gain-of-function mutants provide perhaps the clearest correlation between the ability of syt to engage anionic phospholipid in vitro and its role in driving rapid secretion in neurons. However, other effector interactions (e.g., binding to SNARE proteins) were not examined, so other molecular interpretations are possible.

Another study attempted to address the role of syt-PS interactions (98), but again with the R233Q mutation, which also disrupts the ability of syt to bind to the t-SNARE SNAP-25 (97). Because syt-lipid and syt-t-SNARE interactions are coupled (89, 94), it might not be possible to selectively disrupt the lipid-binding activity of syt via mutations without affecting binding to SNARE proteins. Hence, alternative approaches are needed to address the relevance of each of these interactions. Ideally, a system in which PS can be added or subtracted, without the need to rely on mutations and correlation analysis, would make it possible to test the role of syt-membrane interactions during fusion. These experiments are described in the next section.

**Syt Must Engage Anionic Phospholipids to Regulate SNARE-Mediated Fusion In Vitro**

An important tool in the study of SNARE-mediated membrane fusion is an in vitro fusion assay using reconstituted v- and t-SNARE liposomes. When these liposomes are mixed together, *trans*-SNARE complexes assemble and drive membrane fusion (5, 38, 150, 151). This simple system largely recapitulated the specificity of fusion in multiple membrane-trafficking pathways which had been previously established in yeast, confirming the biological relevance of this approach (38). However, in these studies, SNARE-mediated fusion was not regulated by Ca\(^{2+}\). Tucker et al. (125) addressed this issue by adding the cytoplasmic domain of syt to fusion reactions catalyzed by neuronal SNARE proteins (Figure 5c, d). Apo-syt slightly inhibited fusion, whereas Ca\(^{2+}\)-syt markedly accelerated fusion. These findings are consistent with the emerging data from syt knockout neurons: the dual action of syt to clamp spontaneous fusion in the absence of Ca\(^{2+}\) and to accelerate fusion in response to Ca\(^{2+}\).

The apparent Ca\(^{2+}\) affinity of syt can be enhanced by increasing the fraction of PS used in liposome-binding assays (4), probably because PS helps complete the Ca\(^{2+}\)-binding sites in syt (55, 139). In reconstituted fusion assays that contained the cytoplasmic domain of syt, increasing the level of PS in SNARE-bearing liposomes shifted the Ca\(^{2+}\) dose response to the left, suggesting that PS is a critical effector for the action of syt. In a subsequent study, this idea was tested further: Omission of PS from this assay system had no effect on SNARE-catalyzed fusion but completely abrogated the ability of syt to stimulate fusion in response to Ca\(^{2+}\). This experiment provided the first direct evidence that PS is an essential effector for the action of Ca\(^{2+}\)-syt (Figure 5d) (152). Hence, in vitro fusion assays are emerging as a powerful means to directly assess the function of syt effector interactions during SNARE-mediated membrane fusion (90, 94, 125, 152, 153). Current limitations of the reconstituted fusion system are: (a) the relatively slow kinetics of fusion (125), (b) a lack of Ca\(^{2+}\) sensitivity of membrane-embedded syt (154), and (c) imperfect correlations between loss-of-function mutations studied in cell-based systems (152).
Syt Does “Work” on Membranes

How does Ca\(^{2+}\)-syt regulate fusion by interacting with anionic phospholipids? Because the Ca\(^{2+}\)-binding loops of syt partially penetrate membranes (Figure 5b), they can act, in essence, like cones that—upon insertion—deform the bilayer to drive formation of a localized bulge at the site of vesicle contact (155). Indeed, Ca\(^{2+}\)-syt can do work on membranes, forming tubules and tubulating vesicles (Figure 6b) (90, 134, 138, 156). This “forced curvature” would be expected to facilitate bilayer merger (90, 155, 157). Consistent with this idea, ultrastructural data, for at least some secretory events, indicate that the plasma membrane invaginates toward the secretory organelle (158, 159). However, it should also be noted that a number of proteins, including an RNA polymerase, can effectively tubulate vesicles that contain anionic phospholipids (160).

Do the Ca\(^{2+}\)-binding loops of syt penetrate, and thereby cause local buckling of, the plasma membrane? The linker that connects C2A to the transmembrane domain of syt is flexible enough to allow both cis (i.e., penetration into the vesicle membrane) as well as trans (i.e., penetration into a target membrane) interactions with bilayers (100). Therefore, steering factors, such as the Ca\(^{2+}\)-independent component of binding between syt and PIP\(_2\) (92) or t-SNAREs (discussed in the next section) (70, 161), might bias the cytoplasmic domain of syt so that it penetrates the target membrane in response to Ca\(^{2+}\). This issue has yet to be definitively addressed.

Finally, the membrane penetration/aggregation/tubulation activities of syt are not sufficient to drive fusion in reconstituted systems (90, 94, 152, 153); this conclusion is described below in our discussion of fusion mediated by neuronal versus yeast SNARE proteins. Rather, the simultaneous interaction of syt with membranes and SNARE proteins (Figure 5a), detailed in the following section, appears to be critical for coupling Ca\(^{2+}\) to bilayer merger. It should be noted that some isoforms of syt fail to bind PS altogether (113, 162); these isoforms might function as inhibitory syts (95), akin to i-SNAREs (159), or they might be positive regulators of Ca\(^{2+}\)-independent fusion events (108).

Syt Interacts Directly with SNARE Proteins

In brain detergent extracts, syt is associated with syntaxin as well as with assembled SNARE complexes; these are highly specific interactions, as few other proteins were detected when either syt or SNAREs were immunoprecipitated (70, 161). This argues against the claim that syt and SNAREs are “sticky” and bind proteins indiscriminately, as long as stringent binding criteria are used. These pioneering studies established some degree of binding in the absence of Ca\(^{2+}\). Following these reports, it was shown, using purified recombinant proteins, that binding of the cytoplasmic domain of syt to syntaxin, SNAP-25, assembled t-SNARE heterodimers, or fully assembled SNARE complexes was enhanced by Ca\(^{2+}\) (88, 96, 163–165). Thus, syt can interact with t-SNAREs at all stages of SNARE complex assembly (96). Syt forms direct contacts with t-SNAREs, syntaxin, and SNAP-25 but does not bind to the v-SNARE synaptobrevin (96, 166).

Experiments using full-length recombinant or native syt and SNAREs in detergent micelles have confirmed that binding is promoted by Ca\(^{2+}\) (152, 154, 165, 167). The current debate is whether either of these modes of binding—Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent—is important for exocytosis. One possibility is that the Ca\(^{2+}\)-independent component of binding predisposes syt so that, in response to Ca\(^{2+}\), it can tightly grasp SNARE proteins as it penetrates membranes to carry out physical work on the fusion machinery to initiate exocytosis (124). As alluded to above, Ca\(^{2+}\)-independent binding to t-SNAREs could also steer the membrane-penetration activity of syt toward
the plasma membrane during excitation secretion coupling.

Neither of the isolated C2 domains of syt are able to mediate efficient binding to SNAREs, although some residual binding was observed for the C2B domain (124, 125, 129), and this idea has been confirmed using other methods (88, 89). In contrast, others concluded that the C2B domain was able to fully mediate high-affinity, stoichiometric Ca^{2+}-independent binding (168, 169). This issue was resolved by Tucker et al. (87), who demonstrated that fusing the cytoplasmic or C2B domain of syt to GST enhanced the binding affinity such that efficient complex formation occurred in the absence of Ca^{2+}. Importantly, when t-SNAREs are embedded in membranes and assayed for binding to the cytoplasmic domain of syt, there is complete agreement among all published studies that binding is strongly enhanced by Ca^{2+} (88, 89, 94, 125, 152). The use of full-length membrane-embedded syntaxin is crucial; removal of its transmembrane domain profoundly affects interactions with other proteins (170).

A few studies have begun to map the regions of syt that mediate binding to t-SNAREs. Specifically, substitution of two lysine residues (K326, K327) on the “side” of the C2B domain (Figure 3a) with neutral residues reduced binding (168); complete binding titrations revealed a ~threecold shift in the affinity of this interaction (96). This mutant form of syt exhibited an impaired ability to support regulated exocytosis in the Drosophila NMJ and in autaptic cultures of hippocampal neurons (141, 171, 172). However, this mutation is not specific, as it impairs a number of additional putative effector interactions, including Ca^{2+}-independent binding of syt to PIP2 (92) and the assembly of syt into multimers on lipid monolayers (134).

Substitution of a charged residue in a membrane penetration loop of either C2 domain (Figure 3a) (R233Q in C2A and K366Q in C2B) reduced binding of syt to both membranes and SNAP-25 (97). Because syt can bind to membranes and SNAREs at the same time (94, 124), these data place residues 233 and 366 at the interface between syt, membranes, and SNAP-25 in the fusion complex.

Other studies have addressed the sites on SNARE proteins that interact with syt. It was initially found that syt binds to the H3 domain of syntaxin (124, 163, 174, 175). Importantly, this region of syntaxin assembles into SNARE complexes, so binding of syt could potentially regulate assembly of the fusion apparatus. A subsequent series of NMR studies concluded that the C2A domain of syt instead binds to the N-terminal regulatory domain of syntaxin (called the Habc domain) (57, 176, 177), but these authors have since reported that removal of the Habc domain actually facilitates binding of syt to syntaxin (178), resolving this controversy. Analogous to its interaction with the membrane proximal H3 domain of syntaxin, syt binds to the C-terminal region of SNAP-25 (165, 179) at the base of the SNARE complex near the vesicle and plasma membranes. This binding region is ideally positioned so that syt can interact with membranes while simultaneously driving transitions in the SNARE complex.

Ca^{2+} regulates the interaction of the cytoplasmic domain of syt with SNAP-25 on rapid timescales; binding was complete in ~1.2 ms at μM protein concentrations (96). After binding, a structural transition in the complex was detected, as evidenced by a protein concentration-independent increase in the FRET signal between a donor in syt and an acceptor in SNAP-25. This experiment provided an early indication that syt might drive structural changes in SNAREs.

**Syt Regulates the Structure and Function of SNARE Proteins**

Recent experiments using a reconstituted fusion assay (Figure 5c,d) indicate that the cytoplasmic domain of syt must directly engage...
Figure 7

The tandem C2 domains of syt cooperate to bind membrane-embedded t-SNAREs; Ca\(^{2+}\)-syt directly drives folding and assembly of SNARE complexes. (a) Description of the coflotation assay used to monitor the binding of the cytoplasmic domain of syt to liposomes; bound syt cofloats with liposomes to the top of the gradient. This material is subjected to SDS-PAGE and stained with Coomassie blue. (b) Experimental approach used to monitor binding of syt to membrane-embedded t-SNAREs; these liposomes lack anionic phospholipids (100% phosphatidylcholine, PC), so coflotation can only result from binding of syt to reconstituted t-SNARE heterodimers (syntaxin and SNAP-25). (c) Results from the experiment outlined in panel b, where the cytoplasmic domain of syt cofloated with t-SNARE liposomes by virtue of direct physical contacts with syntaxin and SNAP-25; binding was enhanced by Ca\(^{2+}\). Ca\(^{2+}\)-stimulated binding was not observed for a Ca\(^{2+}\) ligand mutant (CLM) form of syt, in which two critical Ca\(^{2+}\) ligands in C2A and C2B were disrupted by substitutions with neutral amino acids (D230,232N and D363,365N). Binding of isolated C2A was not detected, but isolated C2B exhibited low levels of Ca\(^{2+}\)-stimulated binding activity. These data demonstrate that Ca\(^{2+}\) regulates the interaction of syt with membrane-embedded t-SNAREs and that both C2 domains are required for efficient, stable binding. (d) Assay used to monitor Ca\(^{2+}\)-syt-mediated assembly of SNAP-25, and the cytoplasmic domain of synaptobrevin (cd-syb) onto membrane-embedded syntaxin, which was reconstituted into liposomes in the absence of SNAP-25. (e) Results from the experiment shown in panel d; SNAP-25 and cd-syb failed to efficiently bind to reconstituted syntaxin (5), but addition of Ca\(^{2+}\) and the cytoplasmic domain of syt drove the folding of SNAP-25 onto syntaxin; these t-SNARE heterodimers then efficiently bound cd-syb (181). These results demonstrate that Ca\(^{2+}\)-syt can work on t-SNAREs to alter their structure and function (see also Reference 62). Note: In panel e, the syntaxin liposomes contained PS (25%PS/75%PC) because PS was an essential cofactor that enabled Ca\(^{2+}\)-syt to drive folding of SNARE proteins (94). The data in panel e are from Reference 125; the data in panel e are from Reference 94.
SNARE proteins to drive fusion. Namely, replacement of neuronal SNAREs with yeast SNAREs (which do not physically interact with syt) completely abrogates the ability of Ca\(^{2+}\)-syt to stimulate membrane fusion, despite the fact that syt efficiently penetrates membranes and aggregates liposomes in both systems (94, 153). In addition, truncation of SNAP-25, to mimic cleavage by botulinum neurotoxin A, shifts the Ca\(^{2+}\) requirements for fusion in this assay to the right (125), recapitulating the effect of this toxin on motor neurons (180). Hence, a change in a SNARE protein can directly alter coupling of Ca\(^{2+}\) to fusion, consistent with the idea that SNAREs are relevant effectors of Ca\(^{2+}\)-syt. Finally, mutations in the cytoplasmic domain of syt that selectively reduced Ca\(^{2+}\)-triggered binding to t-SNAREs also impaired Ca\(^{2+}\)-stimulated fusion in vitro (152).

Another experiment, which shed light on the question of whether syt affects SNARE structure and function, made use of the observation that, in reconstituted systems using purified proteins, membrane-embedded syntaxin is unable to efficiently bind soluble SNAP-25; therefore, SNAP-25 added in trans to syntaxin-only liposomes cannot support fusion with v-SNARE liposomes (5). As a consequence, SNAP-25 and syntaxin are routinely purified and reconstituted into liposomes as preassembled heterodimers, which are active and able to drive efficient membrane fusion (5). A key finding was that in the presence of Ca\(^{2+}\), the cytoplasmic domain of syt drove efficient folding of SNAP-25 onto membrane-embedded syntaxin (94); this resulted in the recruitment of the v-SNARE synaptobrevin (Figure 7d,e) (181). Ca\(^{2+}\)-syt-mediated SNARE complex assembly required the presence of PS, indicating that anionic lipids are critical cofactors for the action of syt on SNARE proteins. Finally, once SNAP-25 assembled onto reconstituted syntaxin via the action of Ca\(^{2+}\)-syt, it supported membrane fusion (94). These data unambiguously establish that Ca\(^{2+}\)-syt can alter the structure and function of t-SNAREs.

Before we return to cell-based assays that further explore the function of syt-SNARE interactions, we point out that the small solvable protein, complexin, also interacts with SNARE proteins, but complexin preferentially binds to the assembled SNARE complex (182). It was suggested that syt acts, in part, by displacing complexin from SNARE complexes (166). If complexin serves to block SNARE-mediated fusion, displacement by Ca\(^{2+}\)-syt would allow exocytosis to proceed (183, 184). Arguing against this idea is the finding that disruption of complexin I/II selectively inhibits regulated exocytosis at a step after SV docking and priming, without any effect on the rate of spontaneous fusion (185). If complexin functioned to block SNARE-mediated fusion, an increase in spontaneous release would have been expected (as occurs in syt knockouts, discussed above). Moreover, other studies demonstrated that complexin and syt do not compete for binding to SNARE proteins (182). So, although very high local concentrations of complexin are able to interfere with membrane fusion and exocytosis (166, 184), current data are consistent with the idea that physiological levels of complexin play a positive role in secretion (185, 186).

Functional Analysis of syt-SNARE Interactions in Cells

A handful of studies have sought to address the function of syt-t-SNARE/SNARE-complex interactions in cells. Mutations in SNAP-25, which diminish Ca\(^{2+}\)-triggered binding of syt, had reduced abilities to support exocytosis in PC12 cells (165). In another study, it was reasoned that because the C2 domains of syt must be tethered together to mediate efficient Ca\(^{2+}\)-dependent binding to SNAREs, lengthening the tether that connects them might weaken this interaction. Indeed, lengthening the linker between the C2A and C2B domain of syt diminishes syt-t-SNARE interactions without affecting the on rate, off rate, dissociation rate upon chelation of Ca\(^{2+}\), or the Ca\(^{2+}\) sensitivity of
syt-lipid interactions (96). Expression of these linker mutants in PC12 cells markedly reduced the rate of exocytosis, and (as detailed above in our discussion of fusion pore dynamics) reduced the open lifetime of fusion pores (96). These findings indicate that syt-SNARE interactions regulate the final step in fusion.

Studies from another laboratory resulted in a different view. Namely, by using recombinant C2 domains to inhibit exocytosis in cracked PC12 cells, Shin et al. (187) concluded that phospholipid, but not SNARE binding, is the primary determinant of Ca\textsuperscript{2+}-triggered dense-core vesicle exocytosis. This suggestion was based on two observations: First, the C2A domain of syt-VII binds PS-harboring liposomes at lower [Ca\textsuperscript{2+}] than the C2A domain of syt-I, and second, the C2A domain of syt-VII inhibited release, whereas the C2A domain of syt-I failed to inhibit release. However, at the Ca\textsuperscript{2+} concentrations used to study release in their assay (up to 1 mM), C2A from syt-I and syt-VII bind PS-harboring liposomes to the same extent, and with the same affinity, yet C2A from syt-I fails to inhibit release. Rather, the ability of a C2 domain to inhibit secretion in PC12 cells appears to be due to its ability to bind t-SNAREs and PIP\textsubscript{2}, two molecules that are essential for LDCV fusion (87). An intriguing finding was that the abilities of a panel of C2 domains to bind both t-SNAREs and PIP\textsubscript{2} were strongly correlated (87); perhaps the C2 domains of syts evolved to recognize a PIP\textsubscript{2}·syntaxin complex (142, 188).

It was also reported that Sr\textsuperscript{2+} can drive fast exocytosis without stimulating syt-I-t-SNARE interactions (189), and it was concluded that Ca\textsuperscript{2+}-dependent binding between syt-I and SNARE proteins does not play a role in triggering exocytosis (7). However, the dynamics and action of Sr\textsuperscript{2+} in presynaptic nerve terminals are dramatically different from those of Ca\textsuperscript{2+}, and direct comparisons between these metals are tenuous (190, 191).

Moreover, Sr\textsuperscript{2+} is able to activate some isoforms of synaptotagmin (e.g., syt-VII and -IX and perhaps others), but not syt-I, in reconstituted fusion assays (152). These findings indicate that Sr\textsuperscript{2+} operates in a syt-I-independent manner in neurons, potentially via sensors that mediate asynchronous release.

Adding further controversy is a report that syt-IX cannot bind t-SNAREs at all (192) but can rescue rapid inhibitory synaptic transmission in syt-I null neurons (193). (Note: these inhibitory currents occur over relatively long timescales). Such findings would seem to demonstrate that interactions between syt-IX and SNAREs do not play a role in triggering exocytosis. However, Bhalla et al. (152) demonstrated that syt-IX does in fact bind to t-SNAREs in response to Ca\textsuperscript{2+} and can couple Ca\textsuperscript{2+} to membrane fusion mediated by reconstituted SNARE proteins. Perhaps the lack of binding reported in the earlier study (192) was due to the relatively low levels of syt-IX in brain detergent extracts (152), so that coimmunoprecipitation with SNAREs was not detected.

In summary, most of the published data support a role for syt-SNARE interactions in excitation-secretion coupling. The current challenge is to understand precisely how syt influences the structure of SNARE proteins to regulate transmitter release.

**CONCLUDING REMARKS**

A variety of experimental approaches—ranging from biophysical measurements of transmitter release in neurons and neuroendocrine cells to reconstituted membrane fusion assays—are converging to reveal the function of syt during Ca\textsuperscript{2+}-triggered exocytosis. The emerging view is that apo-syt, which does not have bound Ca\textsuperscript{2+}, inhibits SNARE-catalyzed fusion prior to the Ca\textsuperscript{2+} signal (72, 73, 78, 125); upon binding Ca\textsuperscript{2+}, syt then accelerates SNARE-catalyzed fusion via simultaneous interactions with membranes and SNARE proteins (53, 67, 94, 152). Ca\textsuperscript{2+}·syt carries out physical work on both
lipid bilayers (Figures 5 and 6) and SNARE proteins (Figure 7e,d) and, through these interactions (and potentially others), controls transitions in the fusion machine as a function of intracellular [Ca\(^{2+}\)], including the opening and dilation kinetics of fusion pores (26).

Although significant progress has been made in our understanding of the molecular mechanism of Ca\(^{2+}\)-triggered exocytosis, numerous fundamental questions remain. The structure of the fusion pore itself has yet to be unambiguously determined (Figure 2) (42, 155, 159, 194, 195). The molecular rearrangements that mediate opening and dilation of fusion pores, and precisely how these processes are regulated by Ca\(^{2+}\)-syt, are not yet understood. The answers to these questions will come, in part, from detailed analysis of the core of the regulated membrane fusion machine, which is a complex that contains syt, anionic phospholipids, and SNAREs. The complexity of this machine will increase as additional proteins, which form direct contacts with SNAREs and function at a late step in exocytosis [e.g., complexin (185) and nSec1 (196)], are also incorporated.

Simply stated, it remains to be determined precisely how proteins catalyze membrane fusion. We are now at a stage when we can begin to fit the pieces of this puzzle together and use biophysical approaches to elucidate the inner workings of the membrane fusion apparatus.

### SUMMARY POINTS

1. The localization, structure, and Ca\(^{2+}\)-binding properties of synaptotagmin I (syt) have been determined; both C2 domains of syt are Ca\(^{2+}\)-sensing modules.

2. A new view of the syt null phenotype in mice is emerging: apo-synaptotagmin as a fusion clamp and Ca\(^{2+}\)-synaptotagmin as an accelerator of exocytosis.

3. Detailed mutational analysis of the Ca\(^{2+}\) ligands in syt reveal that some Ca\(^{2+}\)-binding sites appear to be more important for exocytosis than others; the molecular basis for these differences remains unknown.

4. Synaptotagmin regulates fusion pore dynamics and hence controls the final stages of membrane fusion.

5. The molecular mechanism of syt action is as follows: (a) Ca\(^{2+}\)-syt penetrates and does work on membranes, and (b) Ca\(^{2+}\)-syt binds directly to t-SNAREs to influence their structure and function.

### FUTURE ISSUES

1. Does kiss-and-run exocytosis occur at neuronal synapses? If so, is the choice between kiss and run determined by different ranges of [Ca\(^{2+}\)] acting on synaptotagmin? How does Ca\(^{2+}\)-synaptotagmin trigger opening and/or dilation of fusion pores?

2. When, relative to the opening of the fusion pore, do SNAREs assemble into trans-SNARE complexes? Is SNARE complex assembly regulated by Ca\(^{2+}\)-synaptotagmin in vivo?

3. What is the structure of the fusion pore during its initial open state? Is it made of lipids, proteins (i.e., SNAREs), or both? How many SNAREs and synaptotagmins are needed for fusion?
4. How does the membrane-penetration activity of Ca\(^{2+}\)-synaptotagmin regulate the fusion reaction? Which membrane—vesicle or target—must synaptotagmin “dip” into in order to regulate fusion?

5. Why does the disruption of different Ca\(^{2+}\) ligands, within each C2 domain of synaptotagmin, result in different physiological consequences? Why do some of the Ca\(^{2+}\) ligand mutations have no apparent effect on synaptic transmission?

6. In the absence of syt-I, what is the identity of the Ca\(^{2+}\) sensor that regulates asynchronous transmitter release?

7. Is apo-synaptotagmin a fusion clamp that blocks transmitter release—via direct interactions with SNAREs—prior to the Ca\(^{2+}\) trigger? Then, does Ca\(^{2+}\)-synaptotagmin “switch modes” to accelerate fusion via changes in its effector (i.e., membrane and SNARE) interactions?

8. Is it possible to reconstitute Ca\(^{2+}\)-triggered membrane fusion, which occurs on the millisecond timescale, using purified components?

**DISCLOSURE STATEMENT**

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

**ACKNOWLEDGMENTS**

I thank E. Hui for the amperometric traces in Figure 4 and for generating many of the figures shown in this review, A. Bhalla for the data shown in Figure 5d, G. Augustine, M. Jackson, and J. Rizo for permission to reproduce figures from their publications (Figure 3b-d; the bottom panel of Figure 4, Figure 6, respectively). I also thank M. Jackson, J.M. Edwardson, M. Stowell, and members of the Chapman lab for helpful suggestions. This work was supported by grants from the A.H.A. and N.I.H. E.R.C. is an Investigator of the Howard Hughes Medical Institute.

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**Errata**

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