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Abstract: The dyad model was developed to explain the extremely rapid kinetics of synaptic vesicle exocytosis. In contrast to most hypotheses which invoke interactions among synaptotagmins, SNAREs and other regulatory molecules, the dyad model features a quartet of synaptotagmins arrayed at the synaptic vesicle-plasma membrane interface. Ca^{2+} -triggered movements of these synaptotagmins initiate a sequence of events culminating in the fusion of the vesicular and plasma membranes. The relative simplicity of this model and its amenability to empirical testing provide a useful template for future investigations of the molecular events underlying the exocytotic cascade

Dear Dr. Sneyd,

The attached manuscript, "Synaptotagmins 1 and 2 as mediators of rapid exocytosis at nerve terminals: the dyad hypothesis" is submitted for consideration to be published in the Journal of Theoretical Biology. This paper proposes a mechanism by which rapid exocytosis can be driven exclusively via the action of synaptotagmins 1 or 2 which are prominently expressed at fast synapses. In this respect, this model differs from most other hypotheses which implicate SNAREs as the molecules catalyzing membrane fusion at nerve terminals. However, the dyad model is appreciably simpler and more robust than SNARE-based models. Consequently, this paper has the potential to overturn current dogma, and serve as an impetus for a number of decisive empirical investigations. As you can appreciate, there are a number of very prominent groups in the SNARE/synaptotagmin fields, and I would appreciate it if you could avoid selecting as reviewers individuals closely affiliated with the laboratories of Tom Sudhof, Jim Rothman, Reinhard Jahn, Ed Chapman and Jose Rizo. Nevertheless, even excluding this contingent, there are many senior investigators who are qualified to review this submission. Candidates (with email addresses) are:

George Augustine (georgea@neuro.duke.edu)

Richard Tsien (Richard.tsien@nyumc.org)

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Steve Meriney (meriney@pitt.edu)

John Heuser (jheuser@wustl.edu).

Many thanks for your consideration of this submission.

Sincerely,

Cameron Gundersen, Ph.D.

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Highlights

- 1) The dyad model proposes a novel mechanism for the regulation of exocytosis**
- 2) It envisions that synaptotagmin molecules catalyze membrane fusion**
- 3) This model is simpler and more-robust than SNARE models of exocytosis**
- 4) The dyad model makes explicit predictions about the role of synaptotagmins**

Synaptotagmins 1 and 2 as mediators of rapid exocytosis at nerve terminals: the dyad hypothesis

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Abstract

The dyad model was developed to explain the extremely rapid kinetics of synaptic vesicle exocytosis. In contrast to most hypotheses which invoke interactions among synaptotagmins, SNAREs and other regulatory molecules, the dyad model features a quartet of synaptotagmins arrayed at the synaptic vesicle-plasma membrane interface. Ca^{2+} -triggered movements of these synaptotagmins initiate a sequence of events culminating in the fusion of the vesicular and plasma membranes. The relative simplicity of this model and its amenability to empirical testing provide a useful template for future investigations of the molecular events underlying the exocytotic cascade.

Key words: membrane fusion, SNAREs, cysteine string proteins, C2 domains, protein palmitoylation

Introduction

The secretory pathway enables eukaryotic cells to turn over components of the plasma membrane and release substances into the extracellular fluids (Lodish et al., 2012). Animal cells typically have both constitutive and regulated secretory pathways. The distinctive feature of the regulated secretory pathway is that it transforms a signal into the coordinated fusion of secretory organelles with the plasma membrane. Examples of regulated exocytotic secretion include such disparate physiological events as neurotransmitter release, digestive enzyme secretion by the pancreas and the sustained block of polyspermy in eggs. In many cases, the signal that promotes regulated exocytosis leads to a rise of cytosolic ionized calcium (Ca^{2+}) in the target cell. This rise of cytosolic Ca^{2+} is the immediate trigger for the fusion of the membrane of the secretory organelle with the plasma membrane. Because of the crucial cell biological role of regulated secretion, extensive research has delved into the molecular mechanisms underlying this process. Over the last two decades, substantial progress has been made in understanding the role of the proteins that control regulated exocytosis. Based on myriad investigations, a standard model has emerged. This standard model envisions that interactions among a trio of proteins, referred to as SNAREs (soluble, N-ethylmaleimide-sensitive factor attachment protein receptors; Sollner et al., 1993), are the final common pathway for triggering membrane fusion during regulated exocytosis (Weber et al., 1998). Concurrently, the standard model proposes that members of the synaptotagmin (syt) family are the principal elements that regulate the function of the SNARE proteins and thereby allow for the temporal regulation of exocytosis (Jahn et al., 2003; Bai and Chapman, 2004; Jahn and Scheller, 2006; Rizo et al., 2006; Martens and McMahon, 2008; Sudhof and Rothman, 2009; Sudhof and Rizo, 2011). However, many features of this standard model remain to be verified empirically in living cells. Important unresolved issues include the number of SNARE complexes that must assemble to induce membrane fusion, the mechanism by which syts regulate the SNAREs, and

how the energy of SNARE coiling is transmitted to the vesicle-plasma membrane interface to drive membrane fusion.

As an alternative to the standard model, the current paper offers a novel hypothesis for the role of SNAREs and syts in fast, regulated exocytosis. This model is unique, because it proposes that members of the syt family initiate exocytotic membrane fusion without any direct involvement of the SNAREs. This model was developed primarily to explain the extremely rapid (sub-millisecond) triggering of exocytosis that occurs at nerve terminals. Specifically, we hypothesize that the Ca^{2+} -dependent re-distribution of a quartet of syt molecules at the secretory vesicle-plasma membrane interface initiates the membrane fusion event of fast, regulated exocytosis. Owing to the hypothetical arrangement of the syts (Figs. 1-3), we refer to this proposal as the dyad model. A vital feature of the dyad model is that it makes testable predictions that deviate from expectations based on the standard model. Additionally, this model provides insights into events that are left unexplained by the standard models of SNARE-dependent regulated exocytosis. These predictions and explanations will be discussed later. To set the stage for the dyad model, the next sections review important features of membrane fusion and regulated exocytosis.

Results and Discussion

Fusion of lipid bilayer membranes

Fundamentally, exocytosis requires that continuity be achieved between the lumen of a secretory organelle and the interstitial space. This continuity is achieved by the fusion of the secretory organelle membrane and the plasma membrane. Early investigations of membrane fusion, *in vitro*, established that artificial bilayer vesicles prepared from combinations of biologically relevant lipids could be induced to fuse independently of added protein (Papahadjopoulos et al., 1974, 1976; Breisblatt and Ohki, 1976; Duzgunes et al., 1981; Wilschut et al., 1981; Ellens et al., 1985; Deleers et al., 1986). Moreover, Ca^{2+} was observed generally to enhance the rate of vesicle fusion, particularly when anionic phospholipids were

present (Papahadjopoulos et al., 1976; Breisblatt and Ohki, 1976; Duzgunes et al., 1981; Wilschut et al., 1981; Ellens et al., 1985; Deleers, et al., 1986). A key conclusion from these studies is that membrane fusion does not require proteins. This raises an important question: what is the role of protein in membrane fusion in living cells? Most research over the last 30 years indicates that proteins do two things: (i) they regulate the location in the cell *where* exocytosis occurs, or (ii) they regulate *when* exocytosis occurs. Consequently, it is pertinent to ask what has been learned about how proteins regulate the sub-cellular location and timing of exocytosis.

How do proteins control the site(s) of regulated exocytosis?

In general, cells have two overlapping strategies to solve this problem. First, secretory organelles are preferentially transported to specific sub-cellular sites where regulated exocytosis can occur (Lodish et al., 2012). Second, secretory organelles and the target membrane harbor recognition mechanisms that restrict membrane fusion to selected molecular pairings. Indeed, it was investigations of the targeting problem that originally led to the identification and naming of the SNAREs (Sollner et al., 1993), and subsequent studies support the conclusion that SNAREs contribute to the specificity of membrane fusion events (Sudhof and Rothman, 2009). Although this remains an interesting problem, it is peripheral to the main focus of this paper which concerns the events that transpire once secretory organelles reach a suitable location at the plasma membrane.

How do proteins control the timing of regulated exocytosis?

Models of regulated exocytosis need to explain how proteins control the timing of exocytotic events. In this context, two important empirical facts must be kept in mind: first, regulated exocytosis can occur in the sub-millisecond time domain (Katz and Miledi, 1965; Llinas et al., 1981; Sabatini and Regehr, 1996; Martin, 2003). This temporal constraint originally derived from direct measurements of the time delay between the arrival of an action potential at a nerve terminal and the initiation of a post-synaptic

response. These “synaptic delays” were <1 msec (Katz and Miledi, 1965). However, many processes intervene between the depolarization of a nerve ending and the initiation of a post-synaptic response: Ca^{2+} channels open; Ca^{2+} enters the nerve terminal and binds to the exocytotic Ca^{2+} sensor(s); the Ca^{2+} sensor(s) perform(s) some ill-defined task to initiate the fusion of the synaptic vesicle membrane and the plasma membrane; this fusion event leads to the extrusion of neurotransmitter from the synaptic vesicle; the transmitter diffuses post-synaptically where it binds to receptors which transduce the post-synaptic response. Because the duration of several of these steps has since been quantified (Llinas et al., 1981; Sabatini and Regehr, 1996), most investigators infer that the vesicle-plasma membrane fusion event transpires in <0.3 msec (Martin, 2003). To put this in perspective, very few enzymes exhibit catalytic rates exceeding $3,000 \text{ sec}^{-1}$ (Hille). Clearly, exocytotic proteins are capable of acting quickly.

The second empirical fact is that the probability of a secretory organelle fusing with the plasma membrane is profoundly elevated during regulated exocytosis at nerve terminals (Katz, 1969). For instance, while the spontaneous rate of quantal neurotransmitter release at frog motor nerve terminals is about 1 sec^{-1} , this rate can exceed 100 msec^{-1} (a five order of magnitude increase) with the arrival of an action potential (Katz, 1969; Heuser, 1976). Although Ca^{2+} can elevate the rate of fusion of liposomes, it does not increase this rate by orders of magnitude (Duzgunes et al., 1981). These observations support the inference that proteins play crucial roles in controlling both the timing and probability of exocytosis at nerve endings and elsewhere.

To achieve synaptic delays <1 msec, it has been proposed that a strategic distribution of Ca^{2+} channels ensures that the Ca^{2+} needed to trigger exocytosis is delivered effectively to the exocytotic Ca^{2+} sensor(s) (Heuser, 1976; Llinas et al., 1981). Again, although there is evidence that the Ca^{2+} channels at nerve endings are located in close proximity to the sites of regulated exocytosis (Pumplin et al., 1981; Robitaille et al., 1990; Cohen et al., 1991; Wachman et al., 2004), the issue of Ca^{2+} channel localization is

secondary to the development of the current model. Of central importance is the presumption that one or more proteins must initiate membrane fusion by triggering a process that can occur even in the absence of protein. In other words, a key goal is to clarify the role of proteins in converting a low probability event (fusion of pure lipid membranes) into the tightly regulated event seen at vertebrate nerve terminals. The dyad model provides a novel explanation for the role of syts in this process.

A snapshot of the dyad model

The unique feature of this model is that it elevates syts to the pinnacle of the fusion hierarchy for rapid, Ca^{2+} -dependent exocytosis. This model is based on several distinctive structural features of the two major neuronal syts (syts1 and 2; see Figure 1). Because many of these features have not been integrated into previous models of membrane fusion, it is important to specify the prominent regions of these two syts. Starting at the N-end they are: i) intraluminal domain; ii) membrane-spanning domain; iii) palmitoylated cysteine region; iv) juxtamembrane, polybasic region; v) C2A domain; vi) C2B domain. See Fig.1 for details.

The model includes four steps, which will be discussed in detail after this snapshot: 1) Pre-docking: A synaptic vesicle approaches the plasma membrane sufficiently closely that SNARE complex formation can be initiated; 2) Docking: SNAREs engage and form the tri-molecular coiled coils. The free energy of SNARE complex formation contributes to overcoming the electrostatic repulsion between the secretory vesicle surface and the plasma membrane, while helping to stabilize the vesicle at the plasma membrane interface. Thus, SNARE complex formation is primarily a docking step; 3) Priming: next, a priming process prepares the vesicle for fusion. During the crucial phase of priming, a pair of syt dimers is assembled at the vesicle-plasma membrane interface as depicted in Figure 2A. Briefly, the membrane spanning domains of the four syts are induced to converge at the apex of the vesicle-plasma membrane contact zone. This arrangement constitutes the pre-fusion assembly. Four elements of this arrangement

are noteworthy. First, the palmitoylated cysteine region of each syt provides a hydrophobic connector that enables the polypeptide backbone of the syt to emerge from the zone of close apposition between the vesicle and the plasma membrane (Fig.2B). Second, because the palmitoylated cysteine region is presumed to adopt a β conformation, its hydrophobic moieties will project into the outer hemi-bilayer of the docked vesicle and the inner hemi-bilayer of the plasma membrane as shown in Fig.2B. Third, because this cysteine-rich region is implicated in the Ca^{2+} -independent dimerization of syts, the apical syts are shown as pairs with their palmitoylated cysteine regions in close contact as in Fig.2A. Fourth, once the polypeptide backbone of each syt emerges from the zone of vesicle-plasma membrane contact, the juxtamembrane, polybasic region associates electrostatically with anionic membrane lipids of the synaptic vesicle. This organization of both syt dimers ensures that their C2 domains are free to project into the narrow cytosolic gap distal to where the docked vesicle contacts the plasma membrane (Fig.2B and 3A); 4) Exocytosis: Finally, when Ca^{2+} binds to the C2 domains of these apical syts, a cascade of events ensues: The C2A and C2B domains of each syt move from the cytosolic environment and insert into the inner hemibilayer of the plasma membrane (Fig.3B). This movement of the C2 domains is immediately transmitted to the juxtamembrane, polybasic region causing it to dissociate from the surface of the secretory organelle and bind electrostatically to the plasma membrane (Fig.3B). The cumulative impact of the binding to the plasma membrane of both C2 domains along with the juxtamembrane polybasic domain is that the palmitoylated cysteine regions and the membrane-spanning domains of each syt pair are pulled laterally away from the apical contact region between the vesicle and plasma membrane (Figs. 2C and 3B-E). The lateral redistribution and rotation of the membrane-spanning domains of the syts, along with the movement within the opposed hemibilayers of the palmitoylated cysteine residues causes a perturbation of membrane structure at the apical contact between the vesicle and the plasma membrane. This perturbation (shown in a series of steps beginning in Fig. 3C) allows the inner hemibilayer of the vesicle to fold toward the plasma membrane (Figs.3C, D).

Simultaneously, this rotational motion of the syt pairs helps to facilitate an inward dimpling of the outer hemibilayer of the plasma membrane. Together, these protein and lipid re-arrangements culminate in the fusion of the opposed bilayers as illustrated in Figs.3E, F.

Detailed consideration of each step in the dyad model

Steps 1 and 2: Current orthodoxy regards SNAREs as the minimal machinery for a number of membrane fusion events (Weber et al., 1998; Sudhof and Rothman, 2009; Sudhof and Rizo, 2011). The dyad model recognizes the crucial role of SNAREs in the secretory pathway. However, in the present scenario, the energy derived from SNARE coiling does not, per se, lead to exocytotic membrane fusion (note that alternative roles for SNAREs have also been proposed by others; Dunman and Forte, 2003; Martens and McMahon, 2008). Instead, SNARE complex formation is envisioned here as an essential docking step that precedes the assembly of the machinery that enables membrane fusion to be triggered with sub-millisecond fidelity. Theoretical estimates of the energy required to bring bilayer membranes into close apposition suggest that the formation of at least two SNARE complexes is needed for synaptic vesicle docking (Leikin et al., 1987; Rand and Parsegian, 1989; Chernomordik and Kozlov, 2006). Clearly, such energy barriers would be difficult to surmount in the absence of SNAREs and failure of docking would preclude steps 3 and 4 in the dyad model. Nevertheless, it is also important to note that the tight proximity of the secretory organelle and plasma membrane achieved by SNARE coiling almost certainly leads to an increased rate of membrane fusion that is triggered by fluctuations of thermal energy. For instance, in vitro studies have demonstrated that the formation of a single, trans-SNARE complex promotes liposome docking and membrane fusion (Bowen et al., 2004; van den Bogaart et al., 2010). Consequently, it is likely that mechanisms (“fusion clamps”) have evolved to diminish the probability that this SNARE-dependent “proximity effect” leads to membrane fusion in cells that are specialized for regulated exocytosis. Although the molecular identity of these hypothetical fusion clamps remains to be

established, it may emerge that the syts poised at the vesicle-plasma membrane interface can also serve as “fusion clamps” by interfering with lipid interactions that would otherwise drive membrane fusion. This possibility is considered later.

Step 3: The dyad model envisions the assembly of two pairs of syts at the apical contact between a synaptic vesicle and the plasma membrane (Fig.2A). If this model is correct, it is plausible that this arrangement of syt pairs involves other proteins. Specifically, we propose that “chaperone” proteins facilitate this priming event. The possible role of chaperones will be discussed below.

The dyad model also takes into account the evidence that syts1 and 2 form Ca^{2+} -independent dimers, and that this dimerization is due to the cysteine-rich region that follows the membrane spanning domain (von Poser et al.,2000; Fukuda et al., 2001). However, the inter-molecular interactions that mediate this β -mercaptoethanol and SDS resistant dimerization of syts1 and 2 are still not clear, and it will be necessary to demonstrate that these dimers are present, in vivo. Although the dyad model proposes that movements of syt dimers trigger exocytosis, a similar model could be devised using syt monomers.

A novel aspect of the dyad model is that it attributes important contributions to two primary structural features of syts 1and2, the palmitoylated cysteine region and the juxtamembrane polybasic region. The prospective roles of these regions are addressed further: Syts1 and 2 are palmitoylated on the cysteine residues that immediately follow the α -helical, membrane-spanning domain (Fig.1 and Chapman et al., 1996; Veit et al., 1996; Heindel et al., 2003). Indeed, it also appears that the lone cysteine residue (Cys82) of msyt1 and the trio of cysteine residues (Cys90-92) of msyt2 that follow the pair of lysine residues (Lys 90 and 91 in msyt1 and Lys 88 and 89 in msyt2; see Fig.1A) are palmitoylated (Fukuda et al., 2001; Heindel et al., 2003). The structural and functional impact of having a pair of basic residues, which presumably interact with anionic membrane constituents, integrated into this highly hydrophobic

stretch of β -structure remains to be clarified. Nevertheless, it is interesting that msyts1 and 2 both have a T residue preceding the palmitoylated cysteines. This T residue is widely conserved among mammalian syts1 and 2 and may be important for the transition from the α -helical, membrane-spanning domain to the β -structure of the cysteine rich region (T residues are conducive to β structure). Regardless, based on the established sequences of mammalian syts1 and 2 and the evidence for the palmitoylation of the cysteine residues that follow the membrane spanning α -helix, we predict that this 12 residue stretch (73-84 in msyt1 and 81-92 in msyt2) adopts β -structure.

The importance of the proposed 12 residue stretch of β -structure in syts1 and 2 is summarized: As illustrated in Fig. 2B, β -structure allows the membrane-spanning domain of each syt molecule to be poised at the vesicle-plasma membrane interface while the largely hydrophobic palmitoylated cysteine region serves as a “bridge”. This “bridge” enables the distal, hydrophilic regions of each syt molecule to project out into the cytosolic gap between the vesicle and plasma membrane. In addition, because consecutive amino acid residues in this stretch of β -structure will project their R-group at opposing 180° angles, the hydrophobic moieties will be arrayed alternately in the hemibilayers of the apposed membranes (Fig.2B). This alternating distribution of hydrophobic moieties presumably also helps to stabilize the vesicle-plasma membrane contact, or docked state of the vesicle. Evidence for a “stabilizing” role of syts emerged in early studies of *Drosophila* syt mutants (Reist et al., 1998), but the molecular basis of this effect remains to be established.

Next, it is reasonable to ask whether a 12 residue stretch of β -structure is sufficiently long to extend from the apical interface between a synaptic vesicle and the plasma membrane to project out to the hydrophilic environment between the “spherical” vesicle and the plasma membrane (as shown in Fig.2B). Since the average distance between the R groups of the first and third amino acid residue of a β sheet is 0.7 nm, 12 residues of β will extend ~ 3.8 nm. At the same time, if we regard the membrane

spanning α -helix of each syt molecule as a cylinder of ~ 1 nm diameter, then the net distance from the center of the proposed apical cluster of 4 syt molecules (Fig.2A) to the surrounding cytosol is ~ 4.8 nm. Given that the typical diameter of mammalian synaptic vesicles is 40-45 nm, having a radius of contact with the plasma membrane of ~ 4.8 nm is plausible. Unfortunately, systematic measurements of the radius of the contact zone between “primed” synaptic vesicles and the plasma membrane have not been made. Because this issue is germane to membrane fusion models, it will be addressed later.

There is one more issue that needs to be addressed concerning the palmitoylated cysteine region. Fig.1B shows this region embedded in the vesicle membrane with the polypeptide backbone situated in the middle of the bilayer. This inferred location takes into account the fact that it would be energetically prohibitive to have the polypeptide backbone of syt residing at the vesicle surface with the hydrophobic moieties of successive amino acid residues twisted to project into the outer hemi-bilayer of the vesicle membrane. However, once a synaptic vesicle docks at the plasma membrane and syts are moved to the apical interface (as in Fig. 2A, B), there is no energetic barrier to prevent the palmitoylated cysteine region from adopting the interfacial location shown in Fig.2B. Nevertheless, this transition does require flexion at the point where the polypeptide backbone transitions from α -helix to β -structure, and empirical studies will be needed to test whether this flexion occurs. In summary, the palmitoylated cysteine regions of syts1 and 2 are postulated to be important structural and stabilizing elements in the pre-fusion assembly (Figs.2B and 3A, B).

The dyad model also posits a role for the juxtamembrane polybasic region of syts1 and 2. The prediction is that these residues initially interact electrostatically with acidic lipids of the synaptic vesicle surface, thereby extending the tether that projects the hydrophilic C2 domains of each syt into the narrow cytosolic volume adjacent to the vesicle-plasma membrane interface (Fig.2B). Depending on the secondary structure that this tether and the ensuing linker region adopt, the C2A domain should be

situated 4-5 nm from the point of apposition of the vesicle and plasma membranes. Overall, the cumulative contributions of the palmitoylated cysteine and juxtamembrane polybasic regions are to facilitate the organization of the pre-fusion complex as depicted in Figs.2B and 3A.

Step 4: As with all recent models invoking a role for syts in regulated exocytosis, the steps leading to membrane fusion begin with Ca^{2+} binding to the C2 domains of syt. Prominent advances in clarifying how syts help to initiate exocytotic membrane fusion were the findings that Ca^{2+} binding to the C2 domains of several syt family members (including, syts 1 and 2) promotes the partial membrane insertion of these motifs (Bai et al., 2000), as well as membrane bending (Martens et al., 2007). These observations spawned a flurry of biochemical investigations (Bai et al., 2002; 2004; Frazier et al., 2003; Herrick et al., 2006; Bhalla et al., 2006; Martens et al., 2007; Hui et al., 2009) and biological studies (Paddock et al., 2011) of the importance of these phenomena. Concomitantly, numerous hypotheses incorporated the membrane insertion or membrane bending features of syt into exocytotic triggering (Bai and Chapman, 2004; Rizo et al., 2006; Bhalla et al., 2006; Zimmerberg et al., 2006; Martens and McMahon, 2008; Martens, 2010; Paddock et al., 2011). However, none of these proposals placed syt(s) at the apical contact between the synaptic vesicle and the plasma membrane. Thus, a defining feature of the dyad model is the prediction that syts occupy this crucial location and can induce membrane fusion as a consequence of the chain of events discussed next.

In the dyad model, a synaptic vesicle is fully docked and primed when a pair of syt dimers is arrayed as shown in Figs.2A and 3A. Ca^{2+} binding to the C2 domains leads to the movement of the C2 regions of each syt molecule from the cytosolic environment to where they become partially embedded in the plasma membrane. Almost simultaneously, the juxtamembrane polybasic region of each syt molecule dissociates from the vesicle surface and binds electrostatically to the plasma membrane. These paired movements are shown in schematic cross-section in Fig.2B. The rapid and coordinated binding to the

plasma membrane of the polybasic region and the C2 domains is postulated to impose a lateral tugging action on the palmitoylated cysteine region and the transmembrane domains of each syt dimer (Fig.2C). The dyad model hinges critically on the idea that the translocation to the plasma membrane of the C2 and juxtamembrane domains of each syt dimer transmits sufficient force via the polypeptide backbone to cause the lateral separation and rotational pivoting of the transmembrane domains of the syt dimers as shown stepwise in Figs. 3C-F. Obviously, although no work has directly tested the feasibility of this proposal, several lines of evidence bear indirectly on this proposition:

The first consideration concerns whether individual synaptic vesicles harbor enough copies of syt for the dyad model. Current estimates range from 7-15 copies of syt per synaptic vesicle (Takamori et al., 2006; Mutch et al., 2011). Hence, syt copy number is sufficient for the dyad model.

The second crucial matter concerns the minimum lateral separation that must be achieved between the syt dimers to trigger membrane fusion. This distance is directly related to the thickness of biological membranes. Specifically, if the syt dimers in Figs.2C and 3B are pulled apart by a distance equal to the thickness of a biological membrane, then fusion becomes possible. Biological membranes are typically regarded as being ~5 nm thick. Consequently, each syt dimer needs to be displaced laterally a distance of ~2.5 nm. Although there is no definitive information concerning the positioning of the syt C2 domains prior to synaptic vesicle exocytosis, it is plausible that the negative electrostatic potential of the unliganded Ca^{2+} -binding surface of the C2 domains keeps these structures several nm away from the inner hemibilayer of the plasma membrane. If this assumption is correct, then it is reasonable to infer (within the context of Fig. 3B) that the Ca^{2+} -dependent binding to the plasma membrane of the C2 domains transmits vertical and lateral motion to the juxtamembrane polybasic region. The juxtamembrane region then acts as a leash pulling laterally on the hydrophobic elements of the syt dimers thereby driving the required separation and rotation of the syt transmembrane domains (as

shown schematically in Figs.3C-E). This scenario is the central mechanical event in the dyad model, and it provides an explicit mechanism for how a perturbing event can be transmitted to the interface between two membranes that are destined to fuse. In this respect, the dyad model differs from most other hypotheses concerning protein-triggered membrane fusion. We address these differences later.

The third consideration is whether the movement of the syts represented in Figs. 2C and 3B-E provides sufficient perturbation to drive membrane fusion. In the dyad model, the movement of the membrane spanning domains of the syt dimers clearly will alter the vesicle membrane in a direction favoring fusion. However, it is worthwhile considering how the proposed events affect the plasma membrane. The scenario unfolds: First, the rapid lateral movement of the hydrophobic moieties associated with the palmitoylated cysteine region may directly affect the inner hemibilayer of the plasma membrane in a manner conducive to fusion. This would occur because the pair of palmitoylated cysteine regions of each syt dimer would act akin to the bow of a ship slicing through the apposed membrane hemibilayers into which alternating hydrophobic moieties project. This movement should serve both to “zipper” the apposed hemibilayers whilst also dragging membrane lipids in the direction of syt movement. However, the more important consequence of this lateral tugging action is that it will induce a rotation of the membrane-spanning domains of both syt pairs from their initial orientation perpendicular to the plasma membrane to the stage in Fig. 3D where they have pivoted by $\sim 90^\circ$. This rotation of the opposed syt pairs will trigger both an outward bending of the inner hemibilayer of the synaptic vesicle and an inward bending of the outer hemibilayer of the plasma membrane. The outward bending of the inner hemibilayer of the synaptic vesicle membrane is a direct consequence of its association with the hydrophobic transmembrane domains of each syt dimer as they rotate. However, the inward bending of the outer hemibilayer of the plasma membrane is elicited somewhat differently: initially, it reflects the lateral displacement of inner hemibilayer lipids due to the rotational pivoting and lateral movement of the syt dimers (Figs.3C, D). As the syt transmembrane domains rotate, their hydrophobic surfaces then

become templates for interaction with the outer hemibilayer of the plasma membrane (Figs.3C-E). As noted earlier, a lateral movement of each syt pair of ~2.5 nm should be sufficient to drive the events depicted in Figs.3C-E. Given the radius of curvature of synaptic vesicles, once this initial perturbation is underway, release of the inherent membrane tension of the vesicle may be sufficient to drive full membrane fusion. In other words, it is the combination of the lateral, rotational movement of syt dimers and the release of the intrinsic bending energy in the vesicle membrane that presumably drives the final stages of membrane fusion (Fig.3F, G). But, one additional factor may contribute to this dynamic process. During the pivoting of the syt dimers (in the sequence shown in Figs.3C-3E), their intraluminal domains will transit through the nascent fusion pore. Although the overall charge on the intraluminal domains of msyts1 and 2 is negligible, it is noteworthy that the dozen residues closest to the membrane spanning domain include the majority of the intraluminal basic residues (particularly, for syt1). Thus, transient electrostatic repulsion may occur between the syt luminal domains on opposite sides of the developing fusion pore, thereby helping to promote fusion pore expansion. Empirical strategies discussed later should provide insight into this conjecture and the overall plausibility of the dyad model.

The fourth consideration concerns kinetics. Specifically, can the transitions in Fig.3 occur fast enough to mediate synaptic vesicle exocytosis at vertebrate nerve terminals? Although direct measurements of the time needed for the events in Fig.3 currently do not exist, general features of protein dynamics relevant to this situation have been studied empirically (McCammon, 1984). Thus, the time needed for Ca^{2+} binding to the C2 domains, for the short-range (presumably, <5 nm) movements of the C2 domains that lead to membrane insertion and for the membrane dissociation/re-association of each juxtamembrane polybasic region should not exceed 10 μsec (McCammon, 1984). Concomitantly, while the proposed lateral movement of the palmitoylated cysteine regions and the membrane spanning domains of the syt dimers may be impeded by lipid viscosity and collisions with other proteins (the issue of protein

obstacles in the membrane will addressed later), measurements of protein diffusion constants in biological membranes typically give values that are only about tenfold slower than in artificial lipid membranes or in the cytoplasm (Kusumi et al., 2005; Owen et al., 2009). This means that the required ~2.5 nm movement of each syt dimer (Fig.3), can still occur in significantly less than 100 μ sec. Collectively, this leaves ample time for fusion pore dilation and the completion of exocytosis within the allotted 200-300 μ sec. To frame these kinetic estimates in the broader context of membrane fusion models, the dyad model represents a major simplification. For instance, SNARE-based models invoke a series of protein interactions that are appreciably more complicated than the dyad scheme. In addition, SNARE models ignore the issue of how the force generation due to SNARE bundling is transmitted to the membrane hemibilayers that are destined to fuse (Jahn and Scheller, 2006; Rothman and Sudhof, 2009; Sudhof and Rizo, 2011). Consequently, the dyad model provides a simpler and more-robust framework to achieve both the requisite control and rapid kinetics of membrane fusion.

The fifth issue concerns the final steps of the fusion sequence (Fig. 3E-G). As the syts rotate from the position in Fig.3D to Fig.3E, their membrane-spanning domains are shown folding back on the polypeptide backbone. Because steric constraints will limit the extent of this folding, we infer that as tension develops at this pivot point, the α -helixes rotate about their own axes to release the incipient tension. To illustrate this proposed axial rotation, we show the plasma lemmal face of each α -helix as pale blue in Fig.3D. In Fig.3E, the helix has rotated 90° about its axis, so that the pale blue line is centrally situated. In Fig.3F, the 180° of rotation is complete and the pale blue line now faces the merging vesicular membrane. These steps must finally be followed by a reversal of the process that led to the interfacial localization of the palmitoylated cysteine region shown in Fig.2B. In other words, full fusion of the vesicular and plasma membranes must drive the reversion of the palmitoylated cysteine region to an intra-lamellar location as illustrated in Fig. 3G.

Contrasting the dyad model with other models of synaptic vesicle exocytosis

Over the last decade, models of synaptic vesicle exocytosis have emphasized potentially important interactions among the SNARE proteins and syts as well as auxiliary proteins like the complexins and SM proteins (Jahn et al., 2003; Rizo et al., 2006; Bhalla et al., 2006; Zimmerberg et al., 2006; Jahn and Scheller, 2006; Martens and McMahon, 2007; Sudhof and Rothman, 2009; Sudhof and Rizo, 2011). A common feature of these SNARE-centric models is that the fusogenic proteins are arrayed in a ring around the lipid contact between the secretory organelle and the plasma membrane. Because the minimum cross-sectional diameter of the four α -helices that comprise a SNARE bundle is 3-4 nm, there obviously exists a physical constraint that precludes the SNARE complex from occupying the apical contact site proposed for syts in the dyad model (Fig.2B). At the same time, if SNAREs were to occupy this locus, then the SNAREs themselves would be expected to contribute to the formation of the exocytotic fusion pore. Although evidence for this alternative has been advanced (Han et al., 2004), in most models, SNAREs reside external to the contact zone where the lipid constituents of the vesicle and plasma membrane make intimate contact. Because, as noted earlier, the dimensions of this contact region have not been systematically measured, we do not know the diameter of this presumed “ring of SNAREs”. In addition, there is no empirical evidence that addresses how many trans-SNARE complexes (complexes between vesicular and plasma membrane SNAREs) are formed around this ring prior to or during synaptic vesicle exocytosis. Nevertheless, what remains inescapable is that any action exerted by proteins in this ring must be transmitted via some “long-range” force to the lipids at the vesicle-plasma membrane interface. In other words, most membrane fusion models invoke action-at-a-distance. Somehow, the energy released by SNARE coiling, or syt-SNARE interaction is thought to be transmitted laterally from the SNARE ring to the lipids within the vesicle-plasma membrane contact zone. However, with the exception of the scenarios proposed by Zimmerberg, et al. (2006), most models lack explicit treatments of how this force is transmitted to the membrane lipids or the type of molecular re-

arrangements that occur to elicit membrane fusion. In contrast, the dyad model provides a step-by-step sequence of the membrane changes that syt dimers will provoke during fusion pore initiation (Fig.3).

This enables specific predictions to be made and subjected to empirical scrutiny.

What predictions are embodied in the dyad model and how can these predictions be tested?

The dyad model constitutes a significant departure from other membrane fusion models which typically locate the fusogenic proteins in an annulus around the zone of vesicle-plasma membrane contact.

Instead, by exploiting discrete structural elements of syts1 and 2, the dyad model offers a facile mechanism for triggering membrane fusion. Concurrently, it makes a number of empirical predictions:

First, the dyad model postulates that SNARE pairing is a crucial early step in the formation of release-ready synaptic vesicles. Thus, if techniques can be developed to interrogate the status of SNAREs during synaptic vesicle docking, the dyad model predicts that SNARE pairing will precede the assembly of the syt dimers at the vesicle-plasma membrane interface. Concomitantly, if the principal role of SNAREs is to bring vesicles into close contact with the plasma membrane, it may emerge that other proteins stabilize the docked state of the vesicle. In this circumstance, SNARE pairing might be relatively short lived.

However, it would then be necessary to identify other mechanisms that maintain the docked state of the vesicle. It has already been pointed out that the hypothetical arrangement of the syt dimers shown in Fig.2 may represent one of these stabilizing mechanisms. Among the few empirical studies that offer insight into the vesicle-docking issue, there has been speculation that tethers affiliated with synaptic vesicles might represent SNAREs (Harlow et al., 2001; Fernandez-Busnadiego, et al., 2010; Szule et al., 2012). However, the molecular identity of these tethers and the functional state of the vesicles with these tethers remains unclear. Future technological advances should help to resolve this issue.

Second, the dyad model demands the localization of a quartet of syts at the vesicle-plasma membrane interface. In principle, new electron microscopic strategies for localizing proteins (Shu et al., 2011) might

provide morphological evidence in support of this hypothesis. However, the challenge is that only a subset of the docked synaptic vesicles is likely to harbor four syts at this interface. This sampling issue reflects the fact that the “readily releasable” pool of synaptic vesicles is a small fraction of the total population of docked vesicles (Heuser, 1976). Consequently, even if electron microscopic detection of syt were achieved, only a fraction of docked vesicles would be expected to have syts arrayed as shown in Figs.2A and 3A. Thus, although evidence for the apical localization of syts is critical to the dyad model, empirical strategies discussed below may be less cumbersome in assessing this model.

As a complementary strategy to conventional electron microscopy, freeze fracture analysis has the potential to provide data germane to the dyad model. However, in addition to the sampling issue raised in the preceding paragraph, there is the challenge of identifying fracture events that sample the vesicle-plasma membrane interface where primed vesicles should harbor pairs of syt dimers. However, the dyad model also predicts that syts should form part of the wall of the fusion pore during synaptic vesicle exocytosis (Fig.3C-E). If sufficient resolution can be obtained to enable the detection of single, or paired α -helical regions of proteins within the lipid milieu, then it should be possible to determine whether syts form part of the fusion pore during stimulus-evoked exocytosis in preparations such as the frog neuromuscular junction (Heuser, 1976). Alternatively, if procedures for fracture immunolabeling (Rash and Yasumura, 1999) can be extended to syts, then the localization issue can be tested directly.

Third, the dyad model assigns specific functions to structural features of syt that have received scant attention in studies of synaptic vesicle exocytosis. These features include the palmitoylated cysteine region, the juxtamembrane polybasic region, the transmembrane domain and the intraluminal segment. Straightforward predictions can be made for the impact of mutagenesis experiments that target these regions, in vivo. For instance, modifications of the palmitoylated cysteine region may affect exocytosis by altering the dimensions and stability of the vesicle-plasma membrane contact zone, as well as the

proposed zippering of apposed hemibilayers during the early phase of membrane fusion. Alternatively, if the juxtamembrane polybasic region is important for membrane-binding and for transmitting lateral tugging motion to the hydrophobic elements of the syt dimers, suitable mutations in this region should impair or block exocytosis. Similarly, because the transmembrane α -helices of each syt pair are predicted to be critical elements that guide membrane re-organization, manipulation of these domains should affect the kinetics of exocytosis more acutely than in any other membrane fusion model. Lastly, increasing or decreasing the propensity for electrostatic repulsion by the intraluminal domains of syts1 and 2 may subtly affect exocytotic kinetics. Of course, given the crucial role of the C2 domains of syts1 and 2 in exocytotic Ca^{2+} sensing, it will require care to design mutagenesis experiments that do not affect Ca^{2+} sensing, yet distinguish between the tetrad model and other membrane fusion models.

Fourth, although model systems have been widely used to study membrane fusion (Papahadjopoulos, et al. 1974, 1976; Weber, et al. 1998; Fix et al., 2004; van den Bogaart, et al. 2010), it may be challenging to engineer an in vitro system that can approximate the dyad scenario. This is because the dyad model relies on the coordinated action of a pair of syt dimers at the vesicle-plasma membrane interface. In principle, one could begin to mimic this arrangement by incorporating paired syts into liposomal vesicles in a TIRF-based, fusion-monitoring system (Bowen et al., 2004; Fix et al., 2004). However, in practice, these experiments face several obstacles. First, one will need either to use native, purified syt that retains its palmitoylated cysteines, or devise procedures to palmitoylate recombinant syt. Second, it will be necessary to develop a method to assess the number of syt dimers at the liposome-membrane interface. Third, it may be difficult to prevent spontaneous fusion events. Yet, if these obstacles can be overcome and if photolysis of caged Ca^{2+} is used to initiate fusion, then it should be possible to detect and measure the kinetics of syt-driven membrane fusion. Combining this system with mutagenesis studies would offer a powerful complement to the in vivo experiments proposed earlier.

In summary, although experiments can be designed to test the dyad model, it will be challenging to conduct the requisite studies with current technology. Nevertheless, because the dyad model makes predictions that are decisively different from other membrane fusion models, ultimately it should be feasible to discern whether syts1 and 2 function as proposed in Fig.3.

What roles could chaperone proteins and fusion clamps play in the dyad model?

An earlier section alluded to the dramatic impact of nerve impulses to trigger synaptic vesicle exocytosis at frog neuromuscular junctions (Katz, 1969; Heuser, 1976). At the same time, we also know that at frog motor nerve terminals, only a small subset of the morphologically “docked” synaptic vesicles undergoes exocytosis in response to a nerve impulse (Heuser, 1976). These observations have spawned the dual notions that morphologically docked vesicles require further preparation (“priming”) before they become fusion competent, and that once they achieve fusion competence, there may be molecular impediments to fusion (fusion clamps). The following discourse addresses possible priming steps and prospective fusion clamps in the framework of the dyad model.

Priming: Once a synaptic vesicle docks at the plasma membrane, the dyad model proposes that two syt pairs become localized at the vesicle-plasma membrane interface. Although this pairing of syt dimers may occur spontaneously, it is equally reasonable to infer that chaperone proteins contribute to this priming step. Vertebrate nerve terminals have two major families of chaperone proteins, the synucleins and the cysteine string protein (csp)/70 kDa cognate heat shock protein (Hsc70) families (Zinsmaier and Bronk, 2001; Sudhof and Rizo, 2011). Synucleins can associate with synaptic vesicles (Sudhof and Rizo, 2011), and csp- α is an established synaptic vesicle protein (Mastrogriacomo et al., 1994; Buchner and Gundersen, 1997). Moreover, because synucleins have been implicated in managing SNARE complex assembly (Sudhof and Rizo, 2011), csps are better candidates to contribute to syt priming. In fact, csp- α has a distinctive structural motif that makes it almost the ideal candidate to chaperone syts. Specifically,

csp- α is extensively fatty acylated (Gundersen et al., 1994). Most, and possibly all of its cysteine residues are lipid modified. As a consequence, the fatty acylated cysteine string of csp- α can affiliate with the synaptic vesicle membrane in the same manner as shown for the palmitoylated cysteine region of syt1 (syt is in Fig.1B). This arrangement of the polypeptide backbone of csp- α is suited for multiple tasks. It can act as a shepherd to direct or as a barrier to impede the movement of proteins in the plane of the membrane. For the dyad model, we propose that a pair of csp- α monomers initially corral a pair of syt dimers and then facilitate their movement to the vesicle-plasma membrane interface (Fig.4A). Hsc70 may be involved in the recognition and/or translocation phase of this process. Once the syt dimers are delivered apically, we propose that the csps remain at the vesicle-plasma membrane interface and act as barriers to prevent the syt dimers from leaving the contact zone (Figs. 4A, B). Independently, the fact that csp- α , like the syts, can project hydrophobic elements into the opposed hemibilayers (Fig.4B) will further stabilize the vesicle-plasma membrane interface, because the hydrophobic moieties serve as de facto, membrane cross-linking agents. An interesting corollary of this “corral and cross-link function” of the csps is that once the vesicle membrane and plasma membrane are brought into close contact, transmembrane proteins of the vesicle and plasma membrane will no longer have access to this zone of contact. The reason for this exclusion is that there simply is not room for the cytosolic domains of transmembrane proteins to access this interface. Thus, csps are presumed to have multiple roles: they may direct syt pairs to the correct location during priming; they may help to restrain the syt pairs at this interface; they may aid in stabilizing the vesicle-plasma membrane contact zone; and they may directly and indirectly influence the protein constituents within this zone.

The preceding hypothesis concerning the role of csp- α is attractive, because it potentially provides an explanation for the evolutionary conservation of the cysteine string domain of csps (Buchner and Gundersen, 1997). Although a number of different functions have been attributed to csps (Buchner and Gundersen, 1997; Zinsmaier and Bronk, 2001; Sudhof and Rizo, 2011), no light has been shed on the

purpose of the palmitoylated cysteine string. In this context, we speculate that other proteins with fatty acylated cysteine strings (including, the G α -interacting protein, GAIP and the CHIC proteins; DeVries et al., 1996; Cools et al., 2001) may also contribute to the lateral organization or segregation of membrane proteins. Independently, there is biochemical evidence for an interaction between csp- α and syt1 (Evans and Morgan, 2002), which may be relevant to the proposed chaperoning of syts. Additionally, in *Drosophila*, csps have been implicated in modulating the Ca²⁺-dependence of evoked transmitter release (Dawson-Scully et al., 2000), while over-expression of csp- α slows the kinetics of exocytosis in chromaffin cells (Graham and Burgoyne, 2000). The *Drosophila* results could reflect the failure of csp mutants to maintain syts in a fusion competent state, while the over-expression data may be due to an over-abundance of csps at the secretory granule-plasma membrane interface which deter fusogenic syt movements. Independently, an unexplained feature of *Drosophila* csp null mutants is that they exhibit temperature-dependent failure of neurotransmitter release (Umbach et al., 1994). A prediction based on the dyad model is that elevated temperature may destabilize the apical syts of docked and primed synaptic vesicles at nerve terminals of csp null *Drosophila*. Consequently, this system may be useful for testing elements of the dyad model. Clearly, additional work will be needed to assess the proposed roles of csps in vesicle priming and stabilization.

Fusion clamps: The dyad model posits that SNARE pairing serves as a docking step that brings the synaptic vesicle into close contact with the plasma membrane. Based on theoretical treatments of the fusion of lipid bilayers (Gingell and Ginsberg, 1976; Leikin et al., 1987; Chernomordik and Kozlov, 2008), this proximity can lead to membrane fusion via such transient structures as a hemi-fusion intermediate or an inverted hexagonal phase of the apposed membrane lipids. Given that frog motor nerve terminals can have >10⁴ morphologically docked synaptic vesicles (Heuser, 1976) with a spontaneous frequency of exocytotic events of ~1 sec⁻¹ certainly suggests that mechanisms (fusion clamps) exist to diminish the probability of spontaneous fusion events. Although other proteins may contribute as fusion clamps, we

hypothesize that syts1 and 2 also serve as fusion clamps. The reasoning behind this is that once a quartet of syts is deposited at the vesicle-plasma membrane interface (Fig. 2A), it will interfere with the formation of lipid-based fusion intermediates, like the inverted hexagonal phase. Interestingly, a consistent observation from the original studies of syt mutant *Drosophila* was that the frequency of spontaneous exocytotic events was increased at nerve terminals (Broadie et al., 1994; Littleton et al., 1994; Reist et al., 1998). This increase in spontaneous fusion events may be due to the absence or incorrect assembly of apical syts in docked synaptic vesicles.

Like syt, csp- α might also serve as a fusion clamp by disrupting the formation of lipidic fusion intermediates. However, because csps also presumably stabilize the vesicle-plasma membrane interface in a manner that promotes more lipid-lipid contact, their impact on spontaneous fusion events is likely to be more complex. Nevertheless, because both csp- α and syts1and2 are postulated to occupy the critical region where membrane fusion is initiated, it is plausible that other proteins that help to minimize spontaneous fusion events exert their effects in parallel with or via csps and syts.

What data inform us about the dimensions of the contact zone between a synaptic vesicle and the plasma membrane?

In principle, it should be possible to make direct measurements of the dimensions of the contact zone between a synaptic vesicle and the plasma membrane by using electron microscopy. However, two considerations point to the need for caution in making such measurements. First, most electron microscopic images are obtained using chemically fixed tissue. Thus, covalent cross-linking induced by agents, like glutaraldehyde, may artificially increase the zone of contact. Second, electron micrographs are typically presented as projections, and projection artifacts may influence the data set. Technology exists to circumvent both of these concerns, but it has not been applied to the contact zone issue. Specifically, cryopreservation followed by tomographic reconstruction should enable accurate

measurement of the area of vesicle-plasma membrane contact. This combination of approaches was used to study vesicle-associated tethers (Fernandez-Busnadiego et al., 2010), but not the contact zone. Nevertheless, theoretical treatments indicate that an initial membrane-contact area of $\sim 10 \text{ nm}^2$ is sufficient to promote subsequent membrane fusion (Leikin, et al., 1987). In the tetrad model, the contact area is expected to be approximately an order of magnitude larger. Thus, additional work will be required to shed light on this matter.

As noted above, information about the area of vesicle-plasma membrane contact is also important for SNARE-based models of synaptic vesicle exocytosis. This is because this area will define the dimensions of the ring around which SNAREs and any other auxiliary proteins must assemble before performing their physiological role. In this context, it is instructive to consider the ramifications for the biological system of approximating a synaptic vesicle as a rigid sphere (with a radius of 20 nm) contacting a “planar” plasma membrane at a single point. If we assume that the minimum diameter of the four helix bundle of the SNARE complex is 3 nm, then the closest approach that a SNARE complex can achieve to the point of vesicle-plasma membrane contact is 7-8 nm. SNARE complexes cannot come any closer to the contact site, because there is insufficient distance (steric hindrance) between the plasma membrane and the vesicle to accommodate the SNARE bundle. But, vesicle membranes almost certainly are not rigid spheres, so two biological outcomes are plausible. First, one or more SNARE complexes may occupy the point of contact between a vesicle and the plasma membrane. However, because of the 3-4 nm diameter of each SNARE complex, this outcome predicts that release-ready vesicles will remain at least 3-4 nm distant from the plasma membrane with SNAREs sandwiched in between. This distance should be evident in tomographic reconstructions of docked synaptic vesicles and detection of these SNAREs should also be possible by using the approach described by Shu et al., (2011). The other outcome is that the 7-8 nm separation between an assembled SNARE complex and the point of vesicle-plasma membrane contact establishes a plausible boundary for the radius of the SNARE ring. In other words,

once a “spherical” vesicle reaches the point that trans-SNARE complexes can form, the area of contact will be delimited by SNAREs that form bundles 7-8 nm from the point of contact. From these considerations, the theoretical lower limit of the contact zone’s dimensions is $\sim 10 \text{ nm}^2$. The dyad model predicts a minimum radius of contact of $\sim 4.8 \text{ nm}$, giving a contact area of $70\text{-}80 \text{ nm}^2$, while SNARE-mediated docking could produce a contact area as great as $150\text{-}200 \text{ nm}^2$. Clearly, it will be informative to ascertain the biological dimensions of the contact zone for synaptic vesicles.

Concluding comments

The goal of the dyad model is to provide insight into the machinery that enables synaptic vesicle exocytosis to be triggered in the sub-millisecond time domain. As such, it has avoided important questions about the myriad syt gene products which lack the structural elements of msys1 and 2 that are highlighted in this model. For instance, many syts do not effectively bind Ca^{2+} , and thus are unlikely to exhibit membrane insertion/bending (Sudhof, 2002; Martens and McMahon, 2007). Similarly, few of the other vertebrate syt gene products have a region after the transmembrane domain that potentially adopts β -structure as proposed for msys1 and 2. Thus, it will remain an important issue to resolve the function of these other syts. Nevertheless, it is salient that the neuronal syt gene products in *Drosophila* and *C. elegans* exhibit the potential for β -structure following the transmembrane domain, and they both have a polybasic region reminiscent of the region highlighted in Fig.1. Consequently, it is reasonable to speculate that the evolutionary pressure to achieve rapid triggering of neurotransmitter release culminated in the selection of molecular determinants that could mediate this critical process. The relative lack of syt gene redundancy in these invertebrates makes them particularly advantageous for testing elements of the tetrad model.

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FIGURE LEGENDS

Figure 1.

A. The deduced amino acid sequences of mouse syts1 and 2 using the single letter convention.

Structural regions that are featured in the dyad model are highlighted in colors: Red: intraluminal; orange: membrane-spanning α -helix; yellow: palmitoylated cysteine region; green: juxtamembrane polybasic region; blue: includes the C2A domain; purple: includes the C2B domain. The domain boundaries are approximate. For instance, the linker between the two C2 domains is colored for convenience. The same color scheme is used in the representations of syt1 in panel B and in Figs.2-4.

B. Schematic representation of the hypothesized organization of a mouse syt1 monomer in the membrane of a synaptic vesicle (the vesicle membrane is pale pink). The intraluminal domain of syt1 (red) is followed by the membrane-spanning α -helix (orange). The helical region transitions into the proposed 12 residues of β -structure (yellow) which are proposed to be buried in the vesicle membrane. The wavy lines affiliated with the C residues represent fatty acyl modifications of these residues, while ovals of different sizes are associated with the other hydrophobic residues. Lysyl residues have + signs, and the T residue at the start of the β -structure has an -OH. As the polypeptide backbone emerges from the vesicle membrane, it gives way to the juxtamembrane polybasic region (green) which affiliates with the vesicle membrane before linking to the C2A (blue) and C2B (purple) domains. This and subsequent images are intended to illustrate specific features of the dyad model and are not drawn to scale.

Figure 2.

A. This is the proposed organization of paired syt1 dimers at the apical contact between a primed synaptic vesicle and the plasma membrane. In this pre-fusion assembly, the view represented is a cutaway that has removed the plasma membrane so that one is looking at the area of contact where the vesicle has made contact with the inner hemibilayer of the plasma membrane. The membrane spanning domains (red) of the syt pairs are shown as spheres. At the same time, the hydrophobic regions of β -structure (yellow) are shown in the same plane as the top of each helix, because once the syts contact the plasma membrane, the hydrophobic moieties of the β -structure are hypothesized to “cross link” the vesicle and plasma membranes, as shown in Fig. 2B. Moreover, the elements of β -structure are shown in close proximity to one another, because this region apparently participates in the (homo-) dimerization of syts 1 and 2 (see text for further discussion).

B. Hypothesized organization of msyt1 at the interface between a synaptic vesicle and the plasma membrane after assembly of the primed state. This view is a cross section of a single syt molecule. Its partner would sit directly behind it, while the other syt pair would sit to the left. This image exaggerates the proposed role of the region of β -structure (yellow) which is postulated to “cross link” the vesicle and plasma membrane by virtue of the distribution of alternating hydrophobic moieties in the apposed membrane hemibilayers. This schematic also illustrates how this hydrophobic region can serve as a connector that extends from the transmembrane domain (orange) to allow the juxtamembrane polybasic region (green) and the C2 domains to project into the adjacent cytosol.

C. En face representation of the lateral movements of the syt dimers that are proposed to lead to membrane fusion. This is the same view as in A, but after Ca^{2+} binding to the C2 domains of all four apical syts. The binding of Ca^{2+} to the C2 domains leads to their insertion into the plasma membrane

followed by the binding of the juxtamembrane polybasic region (green) to the plasma membrane. These movements are presumed to be transmitted via the palmitoylated cysteine regions (yellow) to the membrane spanning domains (orange) of the apical syt dimers which are shown having moved laterally. Relative to the pre-fusion state in A, this lateral separation of the syt dimers is hypothesized to be the event that initiates membrane fusion via the steps shown in Fig.3. Note that because the C2 domains insert into the plasma membrane and the juxtamembrane region affiliates with the inner hemibilayer of the plasma membrane, these regions are now shown in the same plane as the palmitoylated cysteine segment and the apex of the membrane-spanning domains.

Figure 3

This sequence of images depicts a cross section (side view) of two syt dimers as they transit from the docked and primed state (A) through the sequence of membrane-fusion events triggered by Ca^{2+} entry (in B). In all panels, the plasma membrane is represented as dark pink and the vesicle membrane in lighter pink. The intraluminal domain of each syt is red and the membrane-spanning domain is orange. The alternating residues of the β -structure region are shown as yellow lines linking the apposed membrane bilayers. The lysyl residues in this stretch are denoted by +. The coloring of the juxtamembrane region and C2 domains are as in earlier figures.

A. In the initial, primed state the membrane spanning domains of the syt dimers are in close proximity at the apex of the vesicle-plasma membrane contact. The palmitoylated cysteine region (yellow) serves as a bridge to the juxtamembrane polybasic region (green) which associates with the vesicle membrane, while the C2 domains project into the cytosol.

B. Ca^{2+} binding to the C2 domains triggers a sequence of events that begins with the insertion of the C2 domains into the plasma membrane and the dissociation of the juxtamembrane polybasic region from the vesicle membrane and its association with the plasma membrane. These transitions are represented

by the schematic shifts from A to B. Although the movements of these regions of each pair of syt dimers will be transmitted directly via the polypeptide backbone to the rest of each syt molecule, this image shows only the movements of the C2 domains and the juxtamembrane region immediately before the lateral separation of the transmembrane domains (shown in Fig.2C) commences. It should also be noted that these movements involve domains that account for >75% of the mass of each syt monomer which provides a favorable situation for overcoming the inertial resistance of the hydrophobic elements.

C. The re-distribution of the carboxyl terminal regions of the syt dimers shown in B is postulated to induce a lateral movement and pivoting of the syt dimers. This movement and pivoting is due to the force transmitted through the polypeptide backbone of each syt monomer. As the palmitoylated cysteine regions moves laterally, they will “zipper” the opposed bilayers and tug on the membrane-spanning domains which are shown beginning to pivot. This pivoting of the membrane-spanning domains induces an outward dimpling of the vesicle’s inner hemibilayer and an inward folding of the plasma membrane.

D. Here, the syt membrane-spanning domains have rotated 90° relative to their original orientation in A. This rotation triggers a further outward bending of the vesicle membrane and an inward flexion of plasma membrane. The luminal domain of each syt molecule transiently contributes to the wall of the fusion pore, as the release of the bending energy in the vesicular membrane begins to drive the completion of the exocytotic process in panels E and F. It is important to note that the images shown here correspond to the part of the nascent fusion pore that contains protein. The rest of the developing fusion pore is comprised of membrane lipid that responds to the local perturbation induced by syt movement. In addition, as the syt transmembrane domains continue to rotate toward the extracellular space, they will begin to fold back on the polypeptide backbone of the palmitoylated cysteine region. We propose that the tension that this folding induces is relieved by axial rotation of the α -helix. To

illustrate the direction of this axial rotation (in panels E and F), the surface of the helix facing the vesicular membrane is colored light blue.

E. The continued rotation of the syt dimers propels the luminal domains into the extracellular space as the nascent fusion pore begins to expand to permit the release of the contents of the vesicle. However, this rotation of the membrane-spanning domains also creates tension at the pivot point between the α -helix and the β -structure. We propose that this tension is dissipated by rotation of the helix. Relative to panel D, a rotation of 90° is conveyed by the central blue line in the membrane-spanning α -helix.

F. Compared to panel A, the syt dimers have rotated 180° relative to the membrane axes. At the same time, the membrane spanning domains have swiveled 180° as shown by the blue lines facing the plasma membrane. The final transition to full fusion is for the palmitoylated cysteine domain to revert back to its inter-lamella position. This is shown in panel G.

G. Fusion of the vesicular and plasma membranes is complete. The C2 domains of the syts are shown having dissociated from the plasma membrane (subsequent to the dissociation of Ca^{2+}), while the palmitoylated cysteine region has reverted from its interfacial location to the membrane interior. The crisscrossed gap in the vesicular membrane (pale pink) reflects the addition to the plasma membrane of appreciably more vesicular membrane than is depicted here.

Figure 4

A. As in Fig.2A, this is a cut-away view of the pair of syt dimers at the apex of a docked and primed synaptic vesicle with the syt membrane-spanning domains (red) and palmitoylated cysteine regions (yellow) projecting laterally. The added element in this image is the pair of csp- α molecules (lime green)

which hypothetically chaperone the syts to this location. Only the membrane-associated, cysteine string regions of the csps are represented in this en face view.

B. Proposed membrane association of the fatty acylated cysteine string domain of mouse csp- α .

Although it will be necessary empirically to define the start of the region of β -structure of mouse csp- α , this image starts with F110 and predicts that β -structure is present through the end of the cysteine string. In a fashion similar to what was proposed for syt in Fig.2B, this stretch of β -structure enables csp to project its polypeptide backbone through the region of close contact between a synaptic vesicle and the plasma membrane while also helping to stabilize this interface.

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msyt1: mvsasrpealaapvttvatlvphnatepaspegkedafsklkqkfmnelhkipppwaliaiaivavllvvtccfcvckkclfkknknkkkgkekggknainmkdvkd
msyt2: mrnifkrnqepnvapatttatmplapvapadnstestgpgesqedmfaklkekffneinkiplppwaliaamavvaglllltccfcickkccckknkkekkgkmgkna

msyt1: lgktmkdqalkdddtaetgltdgeekeepkeeeeklgklqysldydfqnnqllvgiiqaaelpaldmggtsdpykvfllpdkkkkfetkvrktlnpvfneqftfkvpysel
msyt2: mnmkdmkggqdddaetgltegegegeeekepenlgklqfsldydfqanqltvgvlqaaelpaldmggtsdpykvfllpdkkkkyetkvrktlnpafnetftfkv

msyt1: ggktlvnavydfdrfskhdiigefkvpmntvdfghvteewrdlqsaekkeqeklgdicfslyvptagkltvvileaknlkkmdvgglspdpyvkihlmqngkrllkkktt
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