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Abstract

Over the last several years, progress in understanding the molecular mechanism of fast, Ca^{2+}-dependent exocytosis at nerve terminals has led to the following textbook picture:The depolarization-dependent opening of presynaptic Ca^{2+} channels leads to a transient elevation of cytosolic Ca ion activity within the vicinity of docked synaptic vesicles. The binding of Ca^{2+} to synaptotagmin 1 (or, 2) then initiates a poorly understood reaction that allows vesicular and target SNAREs (soluble, N-ethylmaleimide-sensitive factor attachment protein receptors) to complete their intermolecular coiling. The energy released by SNARE coiling then drives fusion between the membrane of suitably primed vesicles and the plasma membrane. Although there is widespread agreement that SNAREs and synaptotagmin are crucial for the molecular events that culminate in fast, synchronous exocytosis at neuronal synapses, there remains no consensus about how these proteins trigger the membrane fusion event that is central to this process. This review extends earlier criticisms of models implicating SNAREs in the terminal steps of the exocytotic cascade and explains how recent data are more compatible with a direct role for synaptotagmin 1 (or, 2) in this process.

Key Words: synaptotagmin, syntaxin, synaptobrevin, SNAP-25, membrane fusion, electron tomography

1. Introduction

The goal of this commentary is to take a fresh look at data emerging since the publication of a review [1] which detailed numerous shortcomings of SNARE-based models of fast, synchronous exocytosis at nerve terminals. A theme of the 2014 review was that ongoing research would help to distinguish between SNARE-centric models and a novel,synaptotagmin-mediated mechanism (the dyad model, see ref. 2). Given the hegemony that SNAREs have enjoyed, it is understandable that most research continues to invoke a SNARE-catalyzed fusion event. However, as a counterbalance, this commentary will document how recent observations fit in the framework of the dyad hypothesis and are appreciably more difficult to reconcile with SNARE-based models.

2. The dogma: zippering of SNAREs is a crucial step in catalyzing membrane fusion at nerve terminals.

Nearly all contemporary proposals regarding the final steps of exocytotic membrane fusion rely on contributions of SNARE proteins. The presumed roles for SNAREs are detailed in references 1-11, and the exocytotic cascade unfolds: (i) as a synaptic vesicle approaches the plasma membrane, its v-SNAREs (typically, synaptobrevin 2, syb) attain sufficient proximity that they can engage with cognate t-SNAREs [SNAP-25, for synaptosome-associated protein of 25 kDa and syntaxin 1, syx] on the target (plasma) membrane; (ii) the interaction of the v- and t-SNAREs involves a 1:1:1 stoichiometry among the participants with SNAP-25 contributing two α-helical coils and syb and syx one each to the four helix coil that forms [12-15]; (iii) SNARE "zippering" (as the intermolecular coiling is called) begins at the N-end of each SNARE domain (furthest from the zone of vesicle-plasma membrane contact; Fig.1) and proceeds toward this contact zone with the release of abundant free energy [13,16,17]; (iv) it is hypothesized that the free energy from SNARE zippering is used initially to bring the synaptic vesicle into close proximity to the plasma membrane, and then, via mechanisms addressed later, zippering is thought to be arrested until the arrival of an action potential; (vi) finally, after the depolarization-dependent entry of Ca^{2+} into the nerve terminal, synaptotagmin somehow facilitates full SNARE zippering thereby leading to membrane fusion and the exocytotic release of the vesicle contents.

Based on the preceding scenario, there are three important uncertainties that persist regarding the molecular underpinnings of this process. First, some investigators envision a different pre-fusion organization of SNAREs than the one shown in Fig.1. This alternative is depicted in Fig.2. The subtle difference between the models in Figs. 1 and 2 is that proponents of Fig.1 argue that energy from the completion of SNARE zippering is somehow transmitted to the lipids within the area of vesicle-plasma membrane contact to induce membrane fusion [see ref. 5]. In contrast, by intercalating SNAREs between the vesicular and plasma membranes, advocates of the Fig.2 model infer that SNAREs can induce membrane bending and cause a fusion stalk to form between the vesicle and plasma membrane [3,6-8]. However, both of these models raise substantive concerns that still have not been resolved empirically, and section 4 of this commentary explicitly addresses these concerns.

The second uncertainty has to do with the regulation of the intermolecular coiling of the v- and t-SNAREs. After all, this highly exergonic reaction [13] should be strongly favored as soon as a synaptic vesicle approaches within several nm of the plasma membrane. Given this fact, it immediately became apparent that there needed to be some way to control the onset and/or progression of SNARE zippering. The reason that this control was necessary is that absent a means to arrest SNARE zippering, exocytosis would occur spontaneously each time a synaptic vesicle approached close enough to the plasma membrane for v- and t-SNAREs to engage. Since the resting rate of exocytosis at nerve terminals can be as much as five orders of magnitude lower than the rate achieved during a

"regulated" exocytotic event [discussed in 2,18], it is clear empirically that the underlying control mechanism is highly effective. Fortuitously, investigations conducted in parallel with work on SNAREs revealed that synaptotagmin 1 was the primary Ca^{2+} sensor for synchronous exocytosis at many "fast" synapses [reviewed in ref.1]. This convergence led to the suggestion that synaptotagmin was likely to regulate SNARE-mediated exocytosis by influencing the progression of SNARE zippering. At least five different scenarios have been proposed to explain how synaptotagmin initiates the exocytotic cascade [4], and later sections of this commentary will review evidence that synaptotagmin interacts directly with the SNARE complex. This discussion will also touch on data suggesting that auxiliary proteins, including complexins, munc-13 and munc-18 (mammalian homologs of unc-13 or unc-18) may interface with SNAREs and/or synaptotagmin to regulate the terminal steps of exocytotic membrane fusion.

The third uncertainty concerns the "nitty-gritty details" of just what the energy released by SNARE zippering is doing to bring about membrane fusion. In other words, from the models shown in Figs.1 and 2, it is intuitively evident that once v-SNAREs initiate interactions with t-SNAREs, the nascent formation of the SNARE complex will draw the vesicle membrane into closer contact with the plasma membrane. What remains less obvious is what happens to the opposed membranes as they are drawn into such intimate contact. In some cases, published models of exocytosis ignore the details of how the fusion process unfolds. But, other investigators have developed detailed proposals for the molecular events that may occur at this interface. In section 4, these models will be evaluated in the context of recent EM data and energetic considerations concerning their feasibility.

3. The data: empirical studies that constrain models of exocytotic membrane fusion at nerve terminals

Recent investigations in two areas have yielded findings that need to be accommodated by models of synaptic vesicle exocytosis. The first area is electron microscopy (EM) and the second is multi-faceted investigations that include structural and functional studies of proteins and protein complexes. These data will besummarized below, and their implications for models of exocytosis will be addressed in section 4.

3.1 Electron microscopy (EM)

Technological progress has led to a renaissance in the EM investigation of synapses, and recent results are likely to have a profound influence on our understanding of the molecular events underlying exocytotic membrane fusion. However, before undertaking a review of this work, it is necessary to address some of the procedural concerns that may affect the interpretation of the results: first, all of the studies to be referenced below employed tomographic reconstructions of nerve terminals. These reconstructions are obtained by repeated image acquisition from a series of specimen tilt angles. This approach largely overcomes the "projection artifact" that is inherent in traditional EM images, but it still has limitations. One limitation concerns the algorithm to account for the "missing volume", which is the region of each specimen that is not imaged owing to practical limits on the range of tilt angles. In addition, reliable alignment of successiveimages poses challenges, and there is the potential for specimen degradation during repeated imaging. Collectively, these issues mean that the resolution limit of tomographic studies is in the range of 2-3 nm. This caveat will be revisited later.

The second issue is that there continues to be debate about the best approach for fixing and visualizing biological specimens for EM study. For instance, a recent investigation compared structural parameters for frog motor nerve terminals treated with conventional aldehyde fixatives relative to terminals preserved by rapid freezing [19]. No significant structural differences were observed [19]. Nevertheless, in

addition to the debate about the merits of aldehyde fixation versus cryopreservation, it has been argued that sample dehydration and the use of heavy metals as contrast agents introduces artifacts that are avoided in hydrated, unstained specimens imaged via cryo-EM [20]. At the same time, the cryo-EM approach has intrinsic limitations with respect to the signal: noise ratio for vitrified specimens as well as the meager differences in density (and therefore, contrast) in biological samples [20]. An important contention in the following discussion is that it remains possible that the discrepancy that exists between the structural data reported by the Fernandez-Busnadiego group [20] and many other laboratories (see below) may ultimately be traced to methodological differences. Nevertheless, as outlined later, this is an issue that should be empirically amenable to resolution in the near future.

For the purposes of this review, there are two important questions that have been provisionally answered by EM. The first question concerns the physical location of "release-ready" synaptic vesicles with respect to the plasma membrane. "Release-ready" synaptic vesicles are those vesicles that can undergo exocytosis in response to a single action potential (see ref.1 for further discussion). Specifically, the issue here is whether the membrane of "release ready" vesicles makes direct contact with the presynaptic plasma membrane. This question has assumed increased importance in the aftermath of a report [20] that synaptic vesicles were connected to the plasma membrane via short tethers, but seldom made direct contact with the plasma membrane of resting nerve terminals. This idea that a gap of a few nm persists between synaptic vesicles and the plasma membrane has important implications for understanding the mechanism of fast, synchronous exocytosis at nerve terminals, and specific criticisms of models based on this architecture were presented earlier [1]. The point to be stressed here is that the vast majority of EM studies that used tomographic reconstruction report direct contact between the membrane of a subset of (morphologically, "docked") synaptic vesicles and the plasma membrane. The compilation in Table 1 identifies the organisms that have been studied, the fixation method and the figure(s) in each paper which document contact between the vesicular and plasma membranes. Overall, >15 investigations display images showing no discernible gap between these two membranes (Table 1). Along with the papers cited in Table 1, Rizzoli and Betz (ref.38) reconstructed mouse motor nerve terminals from serial sections and observed synaptic vesicles in contact with the plasma membrane. And, in an important finding, Siksou and colleagues [28] reported that mouse knockouts lacking both munc-13-1 and munc-13-2 were deficient in docked synaptic vesicles relative to wild-type controls. In other words, the absence of two gene products led to a loss of vesicles attached to the plasma membrane. This finding strongly suggests that vesicle:plasma membrane contact is the norm and is not an artifact of tissue preparation. Rather, it reflects the concerted actions of proteins, like muncs-13-1 and -2 that function in the docking process at nerve terminals.

In a separate, influential study [19] systematic measurements were made of the dimensions of the vesicle:plasma membrane contact. These measurements led to the proposal that synaptic vesicles proceed through priming steps that correlate with increasing inter-membrane contact. Another important feature of these observations [19] is that the thickness of the vesicular and plasma membranes at their contact zone is exactly twice the thickness of the unit membranes away from the contact area. In other words, this result indicates that there is no detectable "sandwiching" of any other material between the opposed surfaces. Of course, it remains possible (albeit, implausible) that all of the results cited above and in Table 1 are compromised by methodological issues, and that synaptic vesicles only contact the plasma membrane immediately prior to the fusion event (as suggested by the data in ref. 20). Nevertheless, this apparent disagreement should be amenable to resolution both by more-extensive sampling of nerve terminals by the Fernandez-Busnadiego group along with efforts by other laboratories to replicate their results.

Until then, because of the widespread agreement among far-flung groups that synaptic vesicles directly contact the presynaptic plasma membrane, this outcome will be regarded as the *bona fide* representation of the ultrastructure of nerve terminals. The implications of this conclusion will be addressed in section 4.

As a sidebar to the preceding discussion, the work of Fernandez-Busnadiego and colleagues [20] was restricted to synaptosomes, and it is possible that the physical stress of synaptosome preparation influences the number of synaptic vesicles contacting the plasma membrane. Nevertheless, in a separate study [39] that used serial-section EM to reconstruct synaptosomes, the published images show synaptic vesicles directly abutting the active zone membrane. Thus, it appears that synaptosome preparation does not generally disrupt these inter-membrane contacts.

The second question to be provisionally answered by EM concerns the location and nature of the proteinaceous elements that may contribute to the terminal steps of the exocytotic cascade at nerve terminals. This matter has been most extensively examined in reports from the McMahan laboratory [19, 21, 29, 33, 34] most of which focused on frog, skeletal neuromuscular junctions. This preference for the frog system reflects the repetitive organization of structural elements at this site which facilitates data analysis. In addition, a provocative paper from Cole and co-workers [37] characterized the filamentous (electron-dense) material surrounding synaptic vesicles at nerve terminals of cultured hippocampal neurons. An outline of the relevant results and the authors' interpretation of these data follow:

Beginning with a seminal study in 2001 [21], the frog neuromuscular junction has been subjected to increasingly sophisticated analyses of the structural elements that comprise the presynaptic apparatus. A reasonably stereotyped view has emerged: each active zone has two parallel rows of ~20 synaptic vesicles tethered via "ribs" to a central set of "beams". The ribs, in part, appear to associate with the plasma membrane via "pegs". In addition, the surface of each vesicle opposite its rib contacts is moored to the plasma membrane via "pins" (see Fig.6 of ref.19 for details). Given their location and dimensions, it was recently deduced [19] that the proximal rib segments and pins were likely to represent the SNARE complex (possibly, including accessory proteins). The implications of this assessment will be mooted in section 4.

In a recent EM study [37] of hippocampal nerve terminals, it was found that "docking filaments" ranging from 10-47 nm long and 3-8 nm wide occupied the region of vesicle:plasma membrane contact and projected into the surrounding cytosolic space. Given their dimensions and location, these filaments were regarded as likely to correspond to SNARE complexes (possibly, with accessory proteins). The importance of this observation, namely that macromolecules occupy the interface between "docked" synaptic vesicles and the plasma membrane, will be addressed further in section 4.

3.2 Structural studies of recombinant proteins implicated in regulated exocytosis

A significant boon to our understanding of the roles of proteins in the exocytotic cascade has been high-resolution structural studies of individual proteins, protein complexes or protein fragments. However, it is important to stress two important caveats for all of the structural work published to date. First, these studies have used recombinant proteins that lack the types of post-translational modifications that are found in neurons. For instance, as discussed earlier [1], SNAP-25 and synaptotagmin 1 are palmitoylated on multiple cysteine residues, and this lipid modification will be absent in bacterially produced protein. Thus, it is imperative to keep in mind that the available structural work is only a first approximation of the native structure of these proteins. The second caveat is that the lack of "native" post-translational modifications will very likely influence the types of inter- and intra-protein interactions that can occur for synaptic proteins. Thus, the interactions seen using bacterially produced protein(s) may not be relevant to

events occurring at nerve terminals. The third caveat is that proteins like the SNAREs and synaptotagmins are normally tethered to the surface of a lipid bilayer membrane, and the types of interactions in which they engage are almost certainly constrained by this architecture. To date, most structural work has not addressed this concern. Nevertheless, several papers have emerged recently that deal with interactions among synaptotagmin 1, SNAREs and auxiliary proteins. Before reviewing prominent features of these efforts, the following paragraph takes a brief detour to outline the assumptions that are driving the increasingly detailed and complicated scenarios that continue to be advanced to explain SNARE function at nerve terminals.

A major conundrum that has been the target of myriad recent investigations is the following: if SNAREs are the engines of membrane fusion, and synaptotagmin 1 is the protein that transduces the Ca^{2+} signal, how does "active" (Ca^{2+}-bound) synaptotagmin interface with SNAREs to initiate the fusion cascade? Obviously, the most straightforward explanation is that Ca^{2+}-bound synaptotagmin binds to and activates (or dis-inhibits) SNARE zippering, thereby leading to fusion. However, this relatively simple path has been complicated by other considerations. To begin, there has been the dilemma that SNARE complexes are likely to be involved both in bringing vesicles into close proximity to the plasma membrane, as well as in the terminal stages of membrane fusion. However, rather than propose that there are discrete populations of SNAREs mediating these two separate processes (vesicle "docking" versus membrane fusion), the common inference has been that SNARE complexes go through discrete stages of zippering with the energy from an early zippering step enabling docking while a later zippering step drives fusion. A corollary to this inference has been the recognition that several SNARE-interacting proteins (including, complexins and members of the munc-13 and munc-18 families) may contribute to the regulation of SNARE zippering (see ref. 6 for a detailed assessment of this issue). Consequently, substantial effort has been expended in delineating the interactions among synaptotagmin, the SNAREs and the SNARE-interacting proteins. The following paragraphs review some of the remarkably detailed information that has emerged from such work. However, for reasons discussed in section 4, these investigations tend to create more problems for understanding the terminal steps of the exocytotic cascade than they solve.

Structural and biophysical evidence has accrued that SNARE complex formation (or, dissolution) can occur in discrete steps (40-45). Such data then led to the notion that accessory proteins might stabilize one or more intermediate stages of SNARE zippering (reviewed in ref. 11). The idea that a protein might arrest SNARE zippering was attractive, because this protein could then be displaced (presumably, by synaptotagmin) as part of the exocytotic triggering cascade. Thus, while the munc proteins (munc-13 and munc-18) and their interactions with SNAREs have largely been assigned roles in docking or priming steps of SNARE action (reviewed in ref. 6), complexins emerged as the prime candidates to regulate SNARE zippering prior to the fusion step [40-43]. This focus has led to increasingly detailed structural studies of the association of complexin with the SNARE complex [40-42, 46-49]. These studies have culminated in subtly different models of exocytotic triggering which will be critiqued in section 4.

While the high-resolution structure of native synaptotagmin 1 (or 2) remains a distant goal, recent studies have reported structural analyses of the interaction of soluble synaptotagmin 1 fragments with the SNARE complex [8, 50]. These impressively detailed studies also led to revised models for how synaptotagmin 1 might relieve the complexin-mediated arrest of SNARE zippering thereby leading to membrane fusion. The challenge of reconciling these models with EM data is detailed below.

4. Models of SNARE-driven membrane fusion: do they explain recent data?

The two most common models for explaining the final steps of SNARE-mediated membrane fusion start with the SNAREs deployed as shown in Figs.1 and 2 (see ref.1 for a discussion of other SNARE-based models). There are good reasons for distinguishing between these subtly different models: to begin, in Fig.1, there needs to be a mechanism by which the SNARE complexes that are poised at the perimeter of vesicle-plasma membrane contact communicate with the lipids (or, proteins?) within the contact region to induce membrane fusion. Proposals that aim to explain how further SNARE zippering around the vesicle perimeter might promote fusion are critiqued below. In contrast, the situation in Fig.2 typically envisions full SNARE zippering leading to the coalescence of the opposed lipid bilayers via stalk and hemi-fusion intermediates. The plausibility of this scheme will also be mooted below. The substantive point is that these two different arrangements of the SNARE complexes make very different demands on the fusion machinery which will be addressed in the context of recently published data.

Reconciling Fig.1 models with the data

Based on the results compiled in Table 1, it was concluded in section 3.1 that "release ready" synaptic vesicles make direct contact with the nerve terminal plasma membrane. Thus, fusion models that begin with the situation in Fig.1 obviously are compatible with the EM data in Table 1. However, this organization of the SNAREs raises at least two important questions. First, how do these circumferentially arrayed SNAREs catalyze membrane fusion? Second, if SNAREs really are arranged at the periphery of vesicle-plasma membrane contact, then what are the "electron-dense" elements between the vesicle and plasma membrane described by Cole and colleagues [37]? The following discussion highlights the challenges that these questions pose.

How do the SNARE complexes shown in Fig.1 act to induce membrane fusion?

Although Fig.1 models have appeared in several publications, there are very few details for how these SNAREs promote membrane fusion. One proposal (see Fig.5, ref. 51) is that: "The top diagram displays the sequential priming of synaptic vesicles by partial SNARE/SM protein complex assembly, superpriming by binding of complexin to partially assembled SNARE complexes, and Ca^{2+} triggering of fusion-pore opening by Ca^{2+} binding to synaptotagmin." In another variation on this theme, it was suggested [5] that the "… ''superprimed'' SNARE/SM protein complexes are then substrate for Ca^{2+}-triggered fusion pore opening by Ca^{2+} binding to synaptotagmin, which causes an interaction of synaptotagmin with SNAREs and phospholipids." In a third proposal (ref. 52, Fig.4), it was suggested: "Accordingly, docked synaptic vesicles are most primed when their pins and proximal rib segments are shortest, their vesicle membrane–presynaptic membrane contact areas are largest, their lipid bilayers are most destabilized towards fusion threshold, their associated Ca^{2+}-channels are, on average, in closest proximity to it and they are most eccentric in shape." Alternatively, it has been argued (Striegel et al., 2012) that synaptotagmin has an important role in triggering fusion, because: "Insertion of the hydrophobic residues at the tips of the C2 domains into the core of the presynaptic membrane then triggers fusion by promoting a local Ca^{2+}-dependent positive curvature of the plasma membrane." Clearly, none of these proposals offers molecular details about how the energy from full SNARE zippering or from synaptotagmin interaction with the membrane (or SNAREs) is transmitted to the opposed lipid bilayers to induce membrane fusion. This absence of a unifying explanation of the molecular events that lead to fusion is a major challenge that remains to be addressed in these models. Specifically, there needs to be explicit clarification for why synaptotagmin interaction with SNAREs and phospholipids leads to the opening of a fusion pore, or why vesicles that have the greatest area of contact with the plasma membrane have the highest probability of forming a fusion pore. Obviously, a fusion pore forms. The how and why remain unclear in Fig.1 models.

What is the electron dense material between synaptic vesicles and the plasma membrane, if SNAREs are arrayed external to this interface?

While the model in Fig.1 envisions SNAREs operating outside the vesicle-plasma membrane contact area, Cole and colleagues [37] observed that proteinaceous material appeared to traverse and project out from this interface. Obviously, one possibility (which was favored by Cole and colleagues, ref. 37) is that this material is SNAREs. If this interpretation is correct, it means that SNAREs can be clamped between the vesicle and plasma membrane. There are three of reasons to be skeptical of this interpretation. First, structural work [14,15] reveals that the surface of the SNARE complex harbors a large number of charged amino acid residues. Accommodating a protein complex with this electrostatic charge profile between two phospholipid bilayers will be energetically highly unfavorable, unless there are anionic and cationic lipids that make this arrangement possible. The second concern is that SNARE complexes typically have a cross-sectional profile of 3-4 nm [12,14,15]. If SNARE complexes are clamped between the synaptic vesicle membrane and the plasma membrane, they should either lead to a thickening of the "sandwich" or, if they are pressed into the hydrophobic region of the membrane, then there needs to be a powerful mechanism to neutralize their considerable surface charge. Since we know from the recent measurements [19] that there is no thickening of the "sandwich" where a synaptic vesicle contacts the plasma membrane, the only option that is compatible with observation is that the SNARE complex is "squished" into one or both membrane bilayers. The energy needed to achieve such "squishing" is prohibitive rendering this scenario implausible. The third issue is that if this material represents SNARE complexes, then there needs to be some way for synaptotagmin to communicate with these SNAREs to allow full zippering to take place. Because SNARE zippering proceeds from N- to C-, any region of the SNAREs that protrudes out from the contact area should already be zippered. This makes it very challenging to understand how synaptotagmin can access the region of the SNARE complexes that is buried between the vesicle and plasma membrane. Collectively, these concerns make it highly unlikely that the electron-dense material at the vesicle-plasma membrane interface corresponds to SNARE complexes. However, if this material is not SNAREs, then it becomes imperative to identify this material and integrate it into fusion models. In fact, an alternative proposal for the identity and function of these "electron-dense" elements is given in section 5.

Reconciling Fig.2 models with the data

The most common starting point for SNARE-based models of exocytosis envisions synaptic vesicles poised several nm from the plasma membrane with partially zippered SNARE complexes situated between the vesicle and plasma membrane (Fig.2). Many of these models also incorporate auxiliary proteins (complexin, munc-13 and/or munc-18) along with synaptotagmin into this pre-fusion array. Various proposals have been advanced to explain the molecular events that then culminate in the fusion of the opposed membranes [3, 5-9, 50, 54, 55]. However, the serious challenge that all these models face is that they are not supported by the EM data in Table 1. As discussed in section 3.1, the vast majority of EM findings indicate that the membrane of synaptic vesicles directly adheres to the plasma membrane. There is no gap of 3-4 nm to accommodate SNARE complexes, and certainly, if one needs to introduce synaptotagmin or other SNARE-interacting proteins, this additional protein mass would require volume that simply does not exist at this interface. Globally, this reasoning implies that there is a problem either with the data or the Fig.2 model. The discussion below moots these two possibilities.

If the SNARE model exemplified in Fig.2 is to survive, then the EM data in Table 1 presumably mis-represent the fine structure of nerve terminals. The discussion in section 3.1 points to reasons why the Table 1 data may be suspect. The most compelling argument is that the extensive specimen processing that underlies image acquisition for the studies in Table 1 compromises the results. This consideration was

cited by Fernandez-Busnadiego and colleagues [20] in support of their cryo-EM finding that synaptic vesicles rarely contact the plasma membrane. However, this disagreement can be resolved empirically, so the field should eventually reach a consensus regarding the physical location of a release-ready synaptic vesicle with respect to the plasma membrane. The outcome of such efforts will be decisive for the future of Fig.2 models. However, as noted earlier, most current data argue strongly against the Fig.2 scenario, so it seems unlikely that this view will change. The second consideration is spatial resolution. Specifically, section 3.1 noted that the EM studies in Table 1 have resolution limits of 2-3 nm. However, because the cross-sectional thickness of the SNARE complex is 3-4 nm, one would expect that the studies in Table 1 should have detected a quantifiable gap between synaptic vesicles and the plasma membrane that could accommodate the SNAREs and any other regulatory proteins. Since no discernible gap was evident in the studies in Table 1, it is not a resolution issue that is a challenge for Fig.2 models. Rather, it is their failure to take into account the anatomy that exists at nerve terminals. Thus, the unavoidable conclusion is that fusion models as represented in Fig.2 are not supported by the data and need to be revised accordingly.

Fig. 2 models also have to be reconciled with the observations of Cole and colleagues [37]. The dilemma for Fig.2 models is that electron-dense material transits the area of direct contact between a synaptic vesicle and the plasma membrane [37]. If Fig.2 models are correct, a release-ready synaptic vesicle should sit on a pedestal of electron-dense material that should increase the distance (by at least 3 nm) between the opposed membranes. Since this increased thickness at the site of membrane contact was not observed [19], this prediction is at odds with published evidence. Collectively, these data do not support Fig.2 models.

5. Is there a model of membrane fusion that conforms to recent data?

Yes, the dyad model [2]. Its fusion cascade begins with a quartet of synaptotagmins situated at the interface between a docked synaptic vesicle and the plasma membrane (see the cross-sectional representation in Fig.3). Thus, EM data showing direct vesicle:plasma membrane contact (Table 1) are compatible with the dyad model. Moreover, the dyad model predicts that proteinaceous material should traverse the area of vesicle-plasma membrane contact, as observed by Cole and colleagues [37]. Nevertheless, as outlined below, there remain several opportunities to extend studies in directions that will further distinguish between the dyad model and SNARE-based scenarios.

A characteristic feature of the dyad model is that it exploits relatively unique structural elements of synaptotagmins 1 and 2 which enable these proteins (at least, hypothetically) to be situated at the contact zone between a synaptic vesicle and the plasma membrane [2]. Specifically, there is a 12 residue region of proposed β-structure that immediately follows the membrane spanning α-helix of synaptotagmins 1 and 2. This motif includes several palmitoylated cysteine residues that we [2] argued play a crucial role in enabling the hydrophilic regions of synaptotagmins to project into the cytosolic region adjacent to the vesicle-plasma membrane contact zone (as shown in Fig.3). This arrangement of the synaptotagmins can then be exploited to induce the formation of a fusion pore via a series of steps detailed in ref. 2. An important test of the dyad model will be to integrate mutagenesis studies with the segmentation analysis described by Cole and colleagues [37]. Specifically, the dyad model predicts that suitable mutations that eliminate the β-structure motif (mentioned above) should prevent synaptotagmin from occupying the contact zone between a synaptic vesicle and the plasma membrane. This should lead to a reduction or loss of the "electron dense" material that Cole and colleagues [37] reported at this interface (as well as functional deficits). A similar outcome would be expected for synaptotagmin null mutants. At the same time, mutations of SNARE proteins that do not interfere with vesicle docking should not affect

this "electron dense" material. However, one caveat for the preceding experiments is that cysteine string proteins can also occupy the vesicle: plasma membrane interface (as discussed in ref. 2), so care will be needed in interpreting the EM data. Nevertheless, such studies should help greatly in distinguishing between dyad and SNARE models.

Intrinsic to the data reviewed in section 3.1 is a puzzle that should also help to shed light on the dyad hypothesis. Here is the conundrum: studies of the fine structure of frog nerve terminals by McMahan and colleagues have revealed a wealth of elements (like, ribs and pins) that have been named by virtue of their appearance in segmentation analyses. However, studies of frog nerve terminals have not identified the type of filamentous material at the vesicle: plasma membrane interface reported for rat nerve terminals by Cole and colleagues [37]. What might explain this discrepancy? The trivial answer is that rats are not frogs. However, there is another possibility which could shine a bright light on the molecular transitions that lead up to and culminate in exocytotic membrane fusion at nerve terminals. The argument unfolds: the experimental advantage of the relatively stereotyped appearance of active zones in frog motor nerve terminals is that one can average results over a large number of samples. This averaging then increases the confidence in being able to recognize specific repeating elements, like the ribs and pins. However, the risk of averaging is that one can overlook structures that are present in a (small) subset of the general population. And, this is where the story may get more interesting. The work of Jung and colleagues [19] determined that there was a continuum of areas of contact between a synaptic vesicle and the plasma membrane (from "least primed" to "most primed"). The most-primed vesicles were the ones regarded as having the highest probability of fusing with the plasma membrane in response to an action potential. It will be of considerable interest to determine whether a segmentation study that focuses on the "most-primed" vesicles detects electron dense material at the vesicle: plasma membrane interface. Concurrently, if the "least primed" vesicles lack this material or show a different arrangement of electron-dense material, then there will be even more powerful motivation to pursue the identity of the underlying protein(s) and the molecular transitions that lead to the most-primed state.

6. Conclusions

EM data have been responsible for landmarks in understanding the relationship between structure and function in the nervous system. Early results weighed decisively in favor of chemical versus electrical transmission at most nervous system synapses (discussed in ref. 56). Subsequently, freeze-fracture analyses provided morphological support for the idea that synaptic vesicle exocytosis underlies quantal synaptic transmission (reviewed in ref. 57). Thus, it is appropriate that recent EM findings now constrain models for explaining the molecular details of this signaling process. This review points to new hurdles faced by "textbook" proposals invoking SNAREs in the final stages of exocytotic membrane fusion. Instead, it argues for a simpler scheme that relies on synaptotagmin serving as both the Ca^{2+} sensor for exocytosis and the catalyst of membrane fusion. Although the final illumination of the molecular cascade underlying regulated exocytosis at nerve terminals is unlikely to solve current medical problems, it will lay the foundation for clarifying presynaptic contributions to the full range of nervous system activities, including synaptic plasticity, learning and memory.

Note added in proof.

With respect to the "docking filaments" reported by Cole and colleagues (ref.37),it is evident that a subset of these filaments could correspond to SNAREs.

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Table 1

YearReference	Organism	Fixation	Figure(s) Showing Membrane Contact

2001	21	Frog	Aldehyde	1
2002	22	Lamprey	Aldehyde	4, 5
2006	23	Rat	HPF	2, 4
2006	24	Worm	HPF	1
2006	25	Rat	Aldehyde	1, 3
2007	26	Rat-Mice	HPF	1, 2,3,6,7
2008	27	Rat	Aldehyde	1
2009	28	Mouse	HPF	3, 5
2009	29	Mouse	Aldehyde	1, 3
2011	30	Worm	HPF	1, 3
2011	31	Mouse	Aldehyde	6
2011	53	Fruit fly	Both	2, S5
2012	32	Rat	Aldehyde	1, 2,3,6,8
2012	33	Frog	Aldehyde	1, 3
2013	34	Frog	Aldehyde	2, 3, 8
2013	35	Worm	Aldehyde	2
2014	36	Mouse	HPF	1-6
2016	37	Rat	HPF	1, 4
2016	19	Frog	Aldehyde	2-5

HPF is high pressure fixation.

Figure Legends

Figure 1. SNAREs operate at the periphery of a docked synaptic vesicle.

This figure is a cross-sectional representation of one class of fusion models that begins with the SNARE proteins (syb is blue, syx is red and SNAP-25 is green) arranged circumferentially around the area of contact between the spherical synaptic vesicle and the planar plasma membrane. Although this image is not drawn to scale, it can readily be appreciated that SNAREs operating at these sites must somehow transmit force to the point of vesicle-plasma membrane contact in order to trigger exocytotic membrane fusion. To date, a systematic explanation for how this force transmission occurs and how it induces membrane fusion is lacking. However,

as noted in the text, such models accurately portray the direct contact between vesicular and plasma membranes observed in the studies in Table 1. Nevertheless, they do not help to clarify the identity of the electron-dense material observed at the vesicle:plasma membrane interface in ref. 37.

Figure 2. SNAREs are sandwiched between the synaptic vesicle and the plasma membrane.

The most common hypotheses for how SNAREs initiate exocytotic membrane fusion begin with the organization schematized here (syb is blue, syx is red and SNAP-25 is green). In contrast to Fig.1, the synaptic vesicle is supported on a pedestal of SNAREs and is separated from the plasma membrane by several nm. Although this organization makes it easier to understand how SNAREs can trigger the stalk and hemi-fusion intermediates that are thought to underlie exocytotic membrane fusion (as discussed in detail in refs.1 and 6), these models are incompatible with the EM studies in Table 1 which show synaptic vesicles in direct contact with the plasma membrane.

Figure 3. Proposed location of synaptotagmins in the dyad model of membrane fusion.

In contrast to the scenarios envisioned in Figs. 1 and 2, the dyad model begins with two pairs of synaptotagmins (in purple) arranged at the interface between the synaptic vesicle and the plasma membrane. This cross-sectional image shows opposing members of the synaptotagmin pairs. Then, via the steps described in ref. 2, these synaptotagmins can serve both as Ca^{2+} sensors as well as catalysts of membrane fusion. Crucial advantages of this model are that the synaptotagmins can exist at this interface without disrupting the bilayer structure of the opposed membranes, and they can also be exploited to perturb membrane structure in a manner compatible with fusion pore formation. Clearly, this model is consistent with the data in Table 1 showing direct vesicle:plasma membrane contact, and it predicts that proteinaceous elements will traverse this contact as observed in ref. 37.

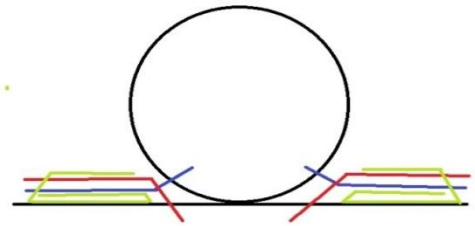

Fig.1

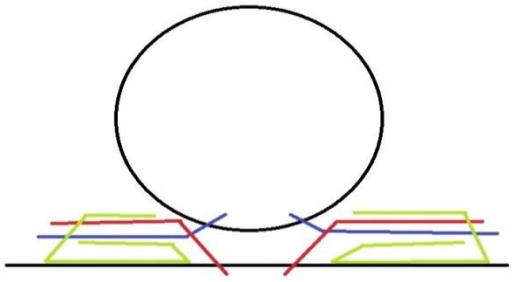

Fig.2

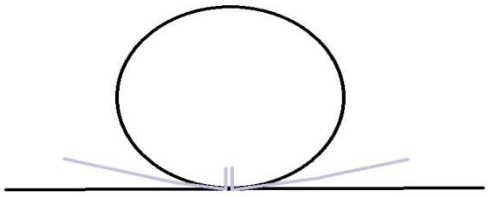

Fig.3

