Title
Fast, Ca2+-dependent exocytosis at nerve terminals: Shortcomings of SNARE-based models

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Abstract: Investigations over the last two decades have made major inroads in clarifying the cellular and molecular events that underlie the fast, synchronous release of neurotransmitter at nerve endings. Thus, appreciable progress has been made in establishing the structural features and biophysical properties of the calcium (Ca2+) channels that mediate the entry into nerve endings of the Ca2+ ions that triggers neurotransmitter release. It is now clear that presynaptic Ca2+ channels are regulated at many levels and the interplay of these regulatory mechanisms is just beginning to be understood. At the same time, many lines of research have converged on the conclusion that members of the synaptotagmin family serve as the primary Ca2+ sensors for the action potential-dependent release of neurotransmitter. This identification of synaptotagmins as the proteins which bind Ca2+ and initiate the exocytotic fusion of synaptic vesicles with the plasma membrane has spurred widespread efforts to reveal molecular details of synaptotagmin's action. Currently, most models propose that synaptotagmin interfaces directly or indirectly with SNARE (soluble, N-ethylmaleimide sensitive factor attachment receptors) proteins to trigger membrane fusion. However, in spite of intensive efforts, the field has not achieved consensus on the mechanism by which synaptotagmins act. Concurrently, the precise sequence of steps underlying SNARE-dependent membrane fusion remains controversial. This review considers the pros and cons of the different models of SNARE-mediated membrane fusion and concludes by discussing a novel proposal in which synaptotagmins might directly elicit membrane fusion without the intervention of SNARE proteins in this final fusion step.

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Dr. Michael J. Zigmond
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April 14, 2014

Dear Michael,

We have enclosed our revised manuscript (PRONEU-D-13-00103) "Fast, Ca^{2+}-dependent exocytosis at nerve terminals: shortcomings of SNARE-based models" for consideration as a review in Progress in Neurobiology. We thank the reviewers for their comprehensive and thoughtful evaluation of our initial submission, and in response to their critique, have edited this version accordingly. Below we outline our response to each of the points raised. We hope you will now find this manuscript suitable for publication in *Progress in Neurobiology*.

Sincerely,

Stephen D. Meriney, PhD
Professor

Responses to Reviewer's criticism:

Reviewer #1
Concern #1: Uneven coverage of topics.
Response: We agree that that some topics are covered with a bit more emphasis than others, and in some locations there are a few more references than in others. We have tried to even this out to some degree, but for some sections (liposome fusion for example), we have the sense that the readers might find it handy to have this collection of references for this topic which is very relevant to the main focus of the review, and as such feel it useful to have these here. On the other hand, for sections like to ones focused on calcium channels, we have made minor modifications, but since this is not the main focus of the review, we have resisted expanding the coverage here so most of our emphasis can be placed on the issues related to vesicle fusion.

Concern #2: Tone down the advocacy for the dyad model and synaptotagmin-centric thought, because this advocacy forces readers to choose between models.

Response: Ultimately, this field must converge on an explanation for how the presynaptic entry of Ca ions triggers the rapid (in <60 □sec) discharge of neurotransmitter from synaptic vesicles. So, yes, at some future point readers will have to choose among models. A central argument of the current review is that the available data do not allow us to distinguish among the exocytosis models that currently exist. A corollary is that techniques are being developed that should help to distinguish among these models, and we tried to emphasize areas where future empirical contributions could make a big impact. Toward this goal, we replaced some of the terms (like, suffer) that this reviewer found to be inappropriate. At the same time, for the simple fact that SNARE models have had the benefit of favorable discussion in dozens of papers and reviews, we did not back off on the extent of coverage of the dyad model. The reason for this is: we could very well have written a review that pointed out the many problems that SNARE models continue to face, and concluded that SNARE models were flawed for the various reasons that were enumerated. However, instead, we tried to be constructive in two ways. First, as noted above, we pointed to several empirical strategies that could help clarify the mechanism of fast exocytosis. And, second, we ended the review by discussing a model that is not burdened by the same problems as SNARE-based models. Our contention is that this "ending-on-a-positive-note" is advantageous in that it will hopefully inspire groups to test elements of the dyad model and eventually generate data that will enrich our understanding of molecular mechanism(s) of exocytosis.

Minor point: This numbering mistake was fixed.

Reviewer #2

In their introductory paragraph, this reviewer outlined two general concerns. These were followed in a second paragraph with very helpful suggestions for diminishing the redundancy between the proposed text box and later sections of the review. Then, there was a long list of specific comments. Our responses are:

First, like reviewer #1, this reader also expressed concern about the extent to which this manuscript focused on the dyad model. As we noted above, our review identifies a number of problems with SNARE models that cannot reconciled with the available data. These concerns often have been ignored in primary research papers and in most reviews of SNARE-based models of exocytosis. Thus, even in spite of the rather lengthy discussion of the dyad model in this review, on balance, discussions of SNARE-based models dominate the literature. The current review is an opportunity to begin to redress this imbalance. By explicitly pointing to empirical strategies that could provide new evidence to discriminate among the exocytotic models that have been proposed, we feel that our coverage of this topic has considerable potential to propel progress in this area by providing a novel alternative to SNARE-based models of exocytosis.

Second, this reviewer asked, "*…is not it quite unlikely that evolution for synaptic vesicle exocytosis would employ SNAREs only for steps in preparation of fusion when sister SNARE isoforms mediate fusion in less-time-critical trafficking events?* " This reviewer raises a very interesting question that hopefully will be addressed in appreciably more detail by evolutionary biologists. Our short answer to this question is: yeast are well known to use SNAREs for membrane fusion events, and these exocytotic events occur very slowly (seconds). However, yeast do not have synaptotagmin-like proteins. Synaptotagmins are found only in multi-cellular

organisms (like, nematode worms) with nervous systems where exocytosis occurs much more rapidly (<1 msec). So, our answer to this question is yes, we think that it is very likely that new machinery has evolved to enable exocytosis to occur as rapidly as it does at mammalian synapses. At the same time, we also pointed out that the dyad model may also be relevant for slower, regulated exocytotic events and that much remains to be done to clarify the role of the synaptotagmins that do not mediate exocytotic Ca sensing.

Third, we arranged to deposit the material in the text box as an online supplement which for most readers will eliminate virtually all the overlap with later segments of the review.

There is one other issue, ribbon synapses, which this reviewer raised repeatedly in the Specific comments. We will tackle this matter here, so that we can refer to the following arguments in our response to some of their "Specific comments". Briefly, we tried to avoid the large and intriguing literature on ribbon synapses, because these synapses clearly operate differently than "non-ribbon" synapses. This difference is perhaps best exemplified by the observations from the Moser group that "conventional" SNAREs do not play a role in exocytotic events at auditory ribbon synapses (Nouvian et al., 2011 Nature Neuroscience 14, 411) and that otoferlin, the presumed Ca sensor in hair cells, and synaptotagmin are not functionally equivalent (Reisinger et al., 2011 J. Neuroscience 31, 4886). From these papers alone, it is safe to infer that the exocytotic machinery at ribbon synapses is sufficiently different from what is envisioned either in standard SNARE-based models or the dyad hypothesis. In fact, the Nouvain paper could stand alone as a major question mark for proponents of SNARE-mediated exocytosis. However, we chose not to address these results. To clarify our focus, we added two sentences (with the above references) to the Introduction to provide the rationale for <u>not</u> discussing exocytotic mechanisms at ribbon synapses.

Specific comments:
1. pgs.4-5, introducing AZ: we have added reference to the concept of a membrane microdomain.
2. pg.5: the Gundelfinger and Fejtova 2012 reference has been added.
3. We have put quotes around the term "active zone precursor vesicle"… is that what the reviewer was requesting?
4. It is not clear to us what the reviewer is requesting with this statement in quotes?
5. The dash in the designation "<alpha>-1" has been removed throughout the document.
6. pg.7: as noted above, we intentionally avoided discussing ribbon synapses.
7. Reference to RIM-BP has been added as suggested, and RIM-BP has been included in Figure 1.
8. Reference added as requested.
9. We eliminated the duplication.
10. We replaced "showed" with "…presented evidence suggesting…."
11. We have moved the Young and Neher reference to more accurately attribute the role of synaptotagmin-calcium channel interactions.
12. For reasons discussed above, we have decided not to include ribbon synapses in this discussion.
13. We were not sure exactly what the reviewer was asking in this comment as it simply quotes one of our sentences without comment. As such, no changes have been made.

14. The statement concerning the low Po of calcium channels during an action potential has been documented by 3 additional references as requested.
15. We agree that the sentence concerning calcium channel gating and the probability of opening during an action potential was not clear. As such, we have edited this section.
16. In section 1.3, as suggested, we have added reference to the importance of active zone size, in addition to the potential number of calcium channel slots.
17. As suggested, the discussion of channel opening during nanodomain coupling has been edited.
18. With respect to using similar figures from previously published work, we do not have a problem using a previously published figure, or a modified figure from a previously published paper in a review article with proper permissions obtained. We feel that this figure displays the concept well and thus prefer to leave it as is.
19. References were fixed as prescribed.

20. pg.22. Add more reference to address SNARE-mediated liposome fusion. Just as there are dozens of papers that address lipid mixing or fusion of pure liposomes that were not cited in this review, we also tried to be selective in citing papers in which SNAREs were involved. The reason for this was that the papers that were cited effectively addressed the main points that we wanted to make: namely, that SNARE-mediated membrane fusion in vitro remains slow relative to what is observed at nerve terminals; that the in vitro assays seldom involve Ca-triggered fusion and in most circumstances use recombinant proteins rather than native proteins for which post-translational modifications have been characterized; and the biologically relevant membrane density of SNAREs remains uncertain. We feel that our concerns are sufficiently well documented in the current references to the primary literature, and consequently, we did not expand the citation list.

21. Criticism of the text box: in general, we concur, and hopefully our solution to this problem (cited above) is adequate. As for FM dye imaging and the work with fluorins, we cited relevant reviews for interested readers.

22. Slow turnover: Yes, we concur with the reviewer on this point which is why the statement addressed “most” rather than “all” proteins.

23. Rather than try to define spatial resolution (as, for instance, Fatt and Katz observed with miniature end plate potentials being detected only “close” to motor end plates), we deleted spatial. We also eliminated the wording that implied that capacitance measurement is not an electrophysiological assay.

24. As noted earlier, for this review’s purposes, ribbon synapses are excluded for the reasons stated above.

25. pg.31 “Among the better known examples of this phenomenon are the” It was not clear to us what the reviewer wanted here, so we made no change.

26. The authors miss on the practice of genetic rescue. We added a phrase in the discussion of knockins (the added phrase is: indeed, knockin of the wild-type gene is often used as a test for the specificity of the KO phenotype) to address this point.

27. Just to clarify our emphasis here: we pointed out that there is ample room for genetic strategies to make further contributions to this field, but we did not take the same stance for peptide work, which appears largely to have run its course. And, in an effort to economize, we had pointed readers to the review by Augustine and colleagues (on page 33) that discussed pros and cons of peptide strategies.

28. The reviewer advocates shortening material on page 40: We took a careful look at this material, but it was not clear that it could be reduced by more than a few words. Because of the different forms of secretion that are reported in the literature, it is necessary to alert the reader to the fact that experimental manipulations have been reported to have differential effects on these different secretory pathways, and it is important to stress that it is not yet clear what precisely these data are telling us. Thus, we did not change this section.

29. We replaced Ca dependence with Ca sensitivity.

30. In spite of the reviewer's overt groan, we kept this sentence to warn readers who may want to jump ahead (as this reviewer clearly wanted to do).

31. We fixed the citation.

32. We added the species in which this work was done.
33. Here, the reviewer states, "*1 ms in the work of Liu et al., 2008 is not slower than sv exocytosis by an order of magnitude*". On the basis of the information the reviewer provided, we respectfully disagree with their assertion. Because we are unaware of any other Liu publication from 2008, we assume that the paper the reviewer is citing is the study in Biophys. J. 94, 1303. The fusion time reported in this work was ~25 msec. Subsequent work from the same two groups [reported in Wang and colleagues (2009) Biophys. J. 96, 4122)], reported that SNARE-dependent fusion events required 5-10 msec. This later paper used a system with better temporal resolution than the 2008 work, but the fusion events were still no faster than 5 msec. As of this point in time (April, 2014), there are NO published reports of SNARE-driven in vitro fusion events that occur any faster than 5 msec. Thus, we stand by our original conclusion, and we did not change the text.

34. To the best of our understanding, this is a comment, and we and the reviewer are in agreement.

35. Yes, we agree with the reviewer that clustering has been observed, but <u>qualitative</u> clustering is very different from a <u>quantitative</u> assessment of protein density that can be expressed as the number of molecules per unit of membrane area. Because it is important for in vitro work to try to achieve quantitative (not, qualitative) agreement with the in vivo situation, we need to know actual numbers. Currently, as we point out, such numbers are lacking. Thus, we made no change.

36. Page 74: work by the Lucic lab: why did we not discuss the reduction of short tethers upon neurotoxin application? The revision now notes that short tethers were reduced after tetanus toxin.

37. Page 73: From what we could tell, this was more of a comment than a criticism/question. We infer that the reviewer wanted us to make sure that readers understand that there can be variability in the release probability for vesicles in the readily releasable pool. To this end, we used the reviewer's wording and added the following parenthetic phrase: it is likely that heterogeneity in release probability exists within the readily releasable pool.

38. Page 80: What do the authors mean by macromolecular template for catalyzing…? The following is a dictionary definition of template:"… a pattern, mold, or the like serving as a gauge or guide in mechanical work." Thus, we envision macromolecules serving as guides for the mechanical work of fusing membranes. We felt that this usage was likely to be sufficiently clear to most readers that it did not require revision.

39. The reviewer was concerned about vagueness in the cited sentence at the beginning of section 2.7. To address this concern, we added the underlined phrase: …certain structural features of synaptotagmins I and II, including the palmitoylated cysteine region and the juxtamembrane polybasic motif (Figure 4)…

40. Page 83: We revised the text to clarify what we meant by "apical" synaptotagmins. Having defined this as clearly as possible, we assume that the reader will follow this argument on the ensuing pages and in the figures. Because the sketch in Fig.4 is meant to highlight the positioning of synaptotagmin molecules at this membrane interface, we felt that adding lipids to the bilayer would distract from the simplicity intended here. Thus, we did not alter the figure.

41. The cited sentence was criticized as being vague and the relevant evidence lacking: The point here is that the dyad model requires that 4 synaptotagmins be arrayed at the (apical) pole where a vesicle makes contact with the plasma membrane. In principle, the 4 synaptotagmins could be recruited to this area after the vesicle makes contact with the plasma membrane, or the 4 synaptotagmins could be pre-arranged on the pole of the vesicle before it reaches the plasma membrane. Because this is a hypothetical matter about which there is no empirical evidence, our discussion referred to the need for experiments to distinguish between these alternatives. Hence, there (currently) is no evidence to discuss.

42. We re-phrased this sentence to avoid this concern about synaptotagmin multimers.

43. The reviewer again takes umbrage at the speculative status of the dyad model and the ensuing comments in this section concerning such issues as the Ca co-operativity of exocytosis. As noted earlier, our defense is that we are aware of no data that "disprove" the dyad model, and at this point the dyad model is consistent with the available data. Moreover, because part of our goal in this review is to encourage the testing of the dyad model, perhaps other readers will be sufficiently "annoyed" to embark on such efforts. Nevertheless, the reviewer makes the excellent point that the issue of Ca co-operativity is further complicated by the fact that C2 domains each

bind multiple Ca ions. In recognition of this point, a phrase was added to the last sentence of this paragraph in section 2.8.4.

Reviewer #3

Page 5: As suggested, we have expanded our discussion of the assembly of the active zone proteins, and further documented the hypothesis that the cytomatrix might arrive first and forms the scaffold around which other proteins are added to the active zone.

Page 7: The suggested references have been added for the cloning of calcium channels.

Page 7: The text has been edited to cite the earlier reference to the "slot" idea that originated in the Tsien lab.

Page 8 and 9: The description of calcium channel subtypes has been unified to refer to both the pharmacological and molecular names in all instances in the text.

Page 23-24: Here, the reviewer points to part of a paragraph which outlines some of the complex functions that "exocytotic" proteins exhibit and suggests that this paragraph could be moved to a different location. We moved this material to section 2.3.
They also asked that references be given for one of the sentences in the paragraph that was to be moved. Because this was an admonition rather than a reference to specific studies, we added "in principle".

Page 24-25: Again, the reviewer felt that the material covered here should be moved to a different location. So, we moved this to material section 2.3 where it follows the material in the preceding comment.

Page 25: Request for the web address of the resource mentioned here: We added the web address.

Page 36: Since we did not include references to some of the earlier enzyme-based efforts, we agree that struggle may be misleading, so we re-worded this phrase.

Page 43: We corrected the typographical errors.

Page 52: The reviewer's comments stem from the observations concerning the severe loss-of-function in mice lacking munc-13 and munc-18 genes. Basically, the reviewer wanted us to go into more depth regarding the implications of these results by adding further discussion either in section 2.8 or 2.9. We added a paragraph in section 2.9 that addresses possible functions of the munc proteins within the dyad framework.

Page 65, Page 66: The reviewer cites two segments of the original text and the asks: "Shouldn't the authors caution that "proximity based fusion of membranes" is also a slow event that may be

a different mechanism from the underlying mechanism of the fast synaptic transmission in vivo?"
We certainly agree with the reviewer on this point (that proximity-based membrane fusion is slow), but we felt that it was unnecessary to re-iterate this point on pages 65 and 66. Since we had already gone into considerable detail on the issue of fusion kinetics a few paragraphs earlier, we felt that this topic was discussed in sufficient detail that it did not need to be reprised here. Thus, no change was made.

Page 67: We added a phrase to elaborate on the progress in fusion assays alluded to here.

Page74-75: We certainly understand the reviewer's perspective on this matter, but from our standpoint, we found the morphological results (of Fernandez-Busnadiego et al., which are under discussion here) to be sufficiently provocative that they deserve the organizational focus used in this paragraph. After all, the argument of the authors who used the unstained, cryo-fixed material is that the precedents in the literature are artifacts of the sample processing procedures employed in earlier studies. Hence, until this contention is thoroughly examined, it will remain unclear whether these criticisms of older technology are correct. Since our goal in this review is to push investigators to undertake experiments that will resolve some of these outstanding issues, we chose not to alter the emphasis of this paragraph (namely, to highlight the reported disparity in morphology).

Page 81: The reviewer asked for further clarification of the content of a sentence dealing with "auxiliary" exocytotic proteins: Since their concern regarding page 52 (above) led to the addition of a paragraph in section 2.9, we now refer the reader to this discussion.

Page 87: A concern and a comment were outlined here:
First, the reviewer wondered how the excess of synaptotagmins on synaptic vesicles (where the data in the literature suggest a range of 7-15 synaptotagmins per vesicle) meshed with the dyad model's requirement for just 4 synaptotagmins. This seems to be an invitation to speculate on conjecture, but in the interest of offering a testable hypothesis, we added fresh commentary in section 2.9. Briefly, our supposition is that although four synaptotagmins suffice for the dyad model, the strong evidence that synaptotagmin plays a role in endocytosis may require that vesicles harbor more copies of synaptotagmin for efficient endocytosis. Again, we stress that this is conjectural and will require a mechanism to alter the stoichiometry of vesicular proteins to be empirically testable, but it does address the reviewer's query.
Second, the reviewer expressed the notion that the dyad model requires cooperation from SNAREs. Yes, SNARE function is embodied in the dyad model. In a nutshell, the dyad model envisions SNAREs bringing membranes into close proximity. Once proximity is achieved, synaptotagmin alone catalyzes the fusion event in response to a Ca signal.

Highlights:

- Presynaptic Ca^{2+} channels regulate fast, synchronous neurotransmitter release
- Synaptotagmins are the main Ca^{2+} sensors for regulated exocytosis at nerve terminals
- SNARE proteins are critical constituents of the exocytotic machinery at nerve endings
- How synaptotagmin regulates SNARE-driven membrane fusion remains controversial
- A new model of synaptotagmin-dependent membrane fusion is discussed

Fast, Ca^{2+}-dependent exocytosis at nerve terminals: shortcomings of SNARE-based models

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Abbreviations: Ca^{2+}, calcium; Rab, ras like in rat brain; RIM, Rab3 interacting molecule; RIM-BP, Rab3 interacting molecule binding protein; CAST, Cytomatrix at the active zone-associated structural protein; MIDAS, metal ion-dependent adhesion site; SNARE, soluble, N-ethylmaleimide-sensitive factor attachment receptor; VAMP, vesicle associated membrane protein; SNAP-25, synaptosome-associated protein of 25 kDa; TIRF, total internal reflection fluorescence; GFP, green fluorescent protein; PKC, protein kinase C; PIP2, phosphatidylinositol bisphosphate; KO, knockout; WT, wild-type.

Key words: Synaptic vesicle fusion; SNARE; Synaptotagmin; Nerve terminal

Abstract

Investigations over the last two decades have made major inroads in clarifying the cellular and molecular events that underlie the fast, synchronous release of neurotransmitter at nerve endings. Thus, appreciable progress has been made in establishing the structural features and biophysical properties of the calcium (Ca^{2+}) channels that mediate the entry into nerve endings of the Ca^{2+} ions that triggers neurotransmitter release. It is now clear that presynaptic Ca^{2+} channels are regulated at many levels and the interplay of these regulatory mechanisms is just beginning to be understood. At the same time, many lines of research have converged on the conclusion that members of the synaptotagmin family serve as the primary Ca^{2+} sensors for the action potential-dependent release of neurotransmitter. This identification of synaptotagmins as the proteins which bind Ca^{2+} and initiate the exocytotic fusion of synaptic vesicles with the plasma membrane has spurred widespread efforts to reveal molecular details of synaptotagmin's action. Currently, most models propose that synaptotagmin interfaces directly or indirectly with SNARE (soluble, N-ethylmaleimide sensitive factor attachment receptors) proteins to trigger membrane fusion. However, in spite of intensive efforts, the field has not achieved consensus on the mechanism by which synaptotagmins act. Concurrently, the precise sequence of steps underlying SNARE-dependent membrane fusion remains controversial. This review considers the pros and cons of the different models of SNARE-mediated membrane fusion and concludes by discussing a novel proposal in which synaptotagmins might directly elicit membrane fusion without the intervention of SNARE proteins in this final fusion step.

Contents

1. Introduction

Nervous systems evolved to mediate rapid intercellular signaling. Two key specializations that underlie this signaling capability are the high-speed conduction of action potentials and fast, synaptic transmission. The last several decades have witnessed major advances in our understanding of the cellular and molecular mechanisms of action potential propagation. At the same time, prominent inroads have been made in clarifying details of the transmitter release cascade and the properties of neurotransmitter receptors. However, important issues remain to be resolved with respect to the machinery that initiates and regulates the fusion of synaptic vesicles with the plasma membrane. This review focuses on outstanding concerns that attend current models of the Ca^{2+}-dependent triggering of neurotransmitter release at "fast" synapses. In this context, it is noteworthy that recent evidence is suggestive of important differences in the molecular machinery for regulated exocytosis at ribbon synapses relative to conventional "fast" (or phasic) synapses (Nouvain et al., 2011; Reisinger et al., 2011). Consequently, this review largely avoids discussion of ribbon synapses. Instead, the first section considers progress in delineating features of the Ca^{2+} channels that regulate the exocytotic discharge of transmitter at phasic synapses, while section two tackles the presynaptic processes that unfold downstream of Ca^{2+} entry.

1.1. Presynaptic Ca^{2+} channels and their role in triggering fast synaptic vesicle fusion

Action potential-triggered chemical transmitter release is an extremely fast process that occurs within one millisecond (Katz, 1969). The presynaptic action potential depolarization that invades the nerve terminal, activates the opening of voltage-gated Ca^{2+} channels, which allow Ca^{2+} ions to enter the nerve terminal according to the electrochemical gradient for Ca^{2+}. Once

inside the nerve terminal, Ca^{2+} ions can bind to an array of Ca^{2+} binding proteins (Rizo and Sudhof, 1998), a subset of which participate in initiating the fusion of the synaptic vesicle membrane with the plasma membrane, and the resulting exocytosis of chemical transmitter molecules (see Walter et al., 2011 for review). In order for this excitation-secretion process to operate in the sub-millisecond time domain, nerve terminals have numerous specializations that contribute to speed, including the close spatial positioning of the proteins that interact in this process.

1.1.1. Assembling transmitter release sites

The region within nerve terminals where Ca^{2+} ions trigger synaptic vesicle fusion with plasma membrane is a membrane microdomain (see Lasiecka et al., 2009 for review) called the active zone. These active zone regions of the nerve terminal are characterized by a collection of specialized proteins that work in concert to facilitate excitation-secretion coupling. The active zone proteins appear to be assembled around a core cytomatrix (see Sudhof, 2012; Gundelfinger and Fejtova, 2012 for review), whose specific location on the cell membrane may be initially defined by the presence of synapse-specific immunoglobin-containing cell adhesion molecules (Shen et al., 2004; Patel et al., 2006; Chao and Shen, 2008; Wanner et al., 2011; Helmstadter et al., 2012). As synapses develop (and remodel), the addition of new active zone cytomatrix components begins with pre-assembly of many of these protein components within the Golgi apparatus into a form of active zone insertion vesicle that can be delivered as pre-packaged components that facilitate rapid assembly of transmitter release sites (Zhai et al., 2001; Ziv and Garner, 2004). The active zone cytomatrix transport vesicles include the active zone proteins piccolo, bassoon, Munc13-1, Rab3 interacting molecule (RIM1α), and ELKS2 (CAST1/ERC2), some of which may be added as these vesicles mature in post-Golgi steps (Schoch and Gundelfinger, 2006; Maas et al., 2012). These “active zone precursor vesicles”, often called Piccolo-Bassoon transport vesicles (PTVs) due to their contents, likely insert at the

nerve terminal plasma membrane as unitary active zone cytomatrix patches, and based on the ability of these proteins to recruit other active zone proteins, these cytomatrix patches form the scaffold around which additional proteins critical for fast transmitter release (which can include SNAREs and Ca^{2+} channels among other active zone proteins) are added, likely via distinct transport vesicles (Ahmari et al., 2000; Zhai et al., 2001; Patel et al., 2006; Regus-Leidig et al., 2008; Owald and Sigrist, 2009). Among the active zone cytomatrix proteins, the RIM family of proteins in particular, appear to be central organizers that bring presynaptic plasma membrane Ca^{2+} channels and vesicle membrane proteins in close proximity to one another (Schoch et al., 2002; Kaeser et al., 2011; Deng et al., 2011; Han et al., 2011). In addition to intracellular macromolecules, the secreted glycoprotein laminin β2 appears to bind Ca^{2+} channels and serves to align pre- and postsynaptic components of the synapse (Nishimune et al., 2004; Chen et al., 2011; Nishimune, 2012). In this manner, the vesicular and plasma membrane machinery needed for fast transmitter release can be spatially organized to enable Ca^{2+}-triggered fusion to occur within the available 60 μs window established by the work of Sabatini and Regehr (1996).

Concurrently, intrinsic membrane proteins of synaptic vesicles are also processed through the Golgi apparatus to form synaptic vesicle protein transport vesicles, which reach the nerve terminal via axonal transport. In fact, these synaptic vesicle protein transport vesicles and the active zone precursor vesicles may traffic together in an aggregate (Tao-Cheng, 2007). As with many Golgi transport vesicles, these often emerge as pleiomorphic vesicles and/or tubulovesicular structures of variable size that appear to require cycling through the nerve terminal plasma membrane before generating mature synaptic vesicles (Hannah et al., 1999; Matteoli et al., 1992, 2004). In particular, vesicle turnover during axonal transport (likely mediated by the exocyst complex of chaperone proteins), may allow a refinement in the distribution of vesicular and plasma membrane proteins that generates a homogeneous pool of competent synaptic vesicles (Murthy et al., 2003). Evidence for this includes studies in

Drosophila where a component of this exocyst complex (sec5) was mutated to prevent vesicle insertion into plasma membrane during axonal transport, which prevented the incorporation of these vesicles into the recycling synaptic vesicle pool at the active zone (without altering synaptic vesicle release carried by previously present synaptic vesicles; Murthy et al., 2003).

Thus, the active zone appears to be assembled in packages by a variety of Golgi transport vesicles that deliver a set of pre-packaged components to the transmitter release site at nerve endings.

1.1.2. Ca^{2+} *channel types and their selective localization at presynaptic active zones*

Voltage-gated Ca^{2+} channels belong to the gene superfamily of voltage-gated ion channels which includes sodium, potassium, and Ca^{2+} channels (Yu and Catterall, 2004). The Ca^{2+} channel family members are distinguished by gene families that encode the pore-forming principle subunit (α1) of these multimeric proteins (Ertel et al., 2000). This α1 subunit is composed of one long (>1,800 residues) amino acid sequence with a predicted membrane topology (based on hydrophobicity plots and experimentally confirmed accessibility of particular amino acids to either extracellular or intracellular compartments) that includes four domains, and each of these domains is composed of six transmembrane segments (Mori et al., 1991; Starr et al., 1991; Williams et al., 1992; Dubel et al., 1992; Fujita et al., 1993). These α1 subunits have generally conserved amino acid sequences that span the plasma membrane, but highly variable regions within the intra- and extracellular loops that confer selective association with other intra- and extracellular proteins. Voltage-gated Ca^{2+} channel diversity and nomenclature has been described (Ertel et al., 2000; Catterall, 2011), and can be further amplified by alternative splicing (Lipscombe et al., 2013), however, only a subset of these Ca^{2+} channel types have been reported to be tightly associated with the transmitter release apparatus at active zones (the Cav2 subfamily of Ca^{2+} channels; see Table 1).

With respect to selective localization of Ca^{2+} channels within active zone regions of the nerve terminal, Tsien and colleagues (Urbano et al., 2003; Coa et al., 2004) proposed that there are a limited number of Ca^{2+} channel "slots" within each active zone (figure 1). By over expressing mutant channels with impaired function, they found that the mutant proteins could compete with endogenous channels for positioning in the active zone. Further, there is evidence that these active zone slots may regulate the types of Ca^{2+} channels that can occupy distinct slot positions (Cao et al., 2010). Thus, one mechanism by which active zones might be organized to enable fast synchronous transmitter release is by restricting specific plasma membrane regions in close proximity to synaptic vesicle release sites for occupancy by discrete Ca^{2+} channel subtypes. The number and position of these locations might be controlled by as yet unidentified extracellular vesicle release site binding proteins (see discussion of α2δ subunits below).

Several mechanisms are thought to be important for the close positioning of Ca^{2+} channels near synaptic vesicle release sites. These include proteins that assist in trafficking Ca^{2+} channels to nerve terminal regions of the neuron, and selective protein-protein binding interactions that may regulate spatial positioning of Ca^{2+} channels within the active zone. Some of the most well studied of these mechanisms are discussed below.

1.1.3. The active zone cytomatrix protein "Rab3 interacting molecule" (RIM)

As described above, the cytomatrix complex that helps organize the active zone proteins includes RIM proteins which have been proposed as central organizers (figure 1). These proteins contain a number of protein interaction domains and may coordinate binding between SNARE proteins, synaptotagmin, Rab3, ELKS, Liprins, RIM binding proteins (RIM-BP), Munc-13, and the voltage-gated Ca^{2+} channel (Sudhof, 2012; Mittelstaedt et al., 2010; Liu et al., 2011). With respect to voltage-gated Ca^{2+} channels, the central PDZ domain in RIM selectively binds only the types expressed in active zones (Cav2 family proteins; primarily Cav2.2 (N-type)

and Cav2.1 (P/Q-type); Kaeser et al., 2011). Further, the C2 domains in RIM may also bind Ca^{2+} channels (Copolla et al., 2001) and modulate channel function (Kiyonaka et al, 2007; Uriu, 2010; Gebhart et al., 2010; Kaeser et al., 2012). Overall, it appears that RIM proteins work in concert with RIM-BP to recruit presynaptic type (Cav2) Ca^{2+} channels to the active zone. This hypothesis is supported by observations in mice and Drosophila using mutant RIM proteins, which document that the interactions of both RIM and RIM-BP with Ca^{2+} channels are essential for channel recruitment to the active zone (Kaeser et al., 2011; Liu et al., 2011a). Lastly, a conditional knockout of RIM proteins in the mouse calyx of Held synapse resulted in a reduction in Ca^{2+} channel density in these nerve terminals, further suggesting that RIM proteins are essential for enriching these transmitter releasing regions with Ca^{2+} channels (Han et al., 2011). Interestingly, for those channels that did remain in the nerve terminal, the lack of RIM proteins slowed the kinetics of Ca^{2+}-triggered transmitter release, which, based on a calculation of the Ca^{2+} concentration near synaptic vesicles, was interpreted to indicate that there was a greater average distance between presynaptic Ca^{2+} channels and docked synaptic vesicles (a loosening of the Ca^{2+} channel-vesicle coupling; Han et al., 2011). Therefore, RIM proteins are important regulators of Ca^{2+} channel targeting to active zone regions of the nerve terminal, and tight coupling to synaptic vesicle release sites.

1.1.4. The synprint site

Rettig and colleagues (1996) identified an amino acid sequence that spans the intracellular loop between domains II and III of the α1 subunit within specific presynaptic subtypes of Ca^{2+} channels (Cav2.1 and Cav2.2), and interacts with the SNARE proteins syntaxin and SNAP-25. They termed this amino acid sequence the synaptic protein interaction site (the “synprint” site; see Table 1). Mochida and co-workers (1996) showed that synthetic synprint peptides could compete with Ca^{2+} channels for binding to SNARE proteins within cultured superior cervical ganglion synapses. The introduction of these peptides reduced the

magnitude of action potential-triggered synchronous transmitter release, which was interpreted to be due to drifting of presynaptic Ca^{2+} channels away from vesicle release sites after their normal binding to SNARE proteins was inhibited by competition with synprint peptides. This synprint site was also shown to mediate binding between Ca^{2+} channels and synaptotagmin, the Ca^{2+} sensor for transmitter release (Kim and Catterall, 1997; Sheng et al., 1997; 1998; Young and Neher, 2009). Therefore, it appears that one mechanism by which voltage-gated Ca^{2+} channels can closely associate with synaptic vesicles within active zone regions of the nerve terminal is through a direct binding to SNARE proteins and synaptotagmin. However, many features of this interaction remain to be resolved.

The binding between presynaptic Ca^{2+} channels and the SNARE protein syntaxin has also been shown to modulate the function of Ca^{2+} channels, such that when bound by the closed configuration of syntaxin (which occurs in the absence of vesicle docking and SNARE protein coiling), Ca^{2+} channels were less likely to open during a depolarization (Bezprozvanny et al., 1995; Wiser et al., 1996; Degtiar et al., 2000; Bergsman and Tsien, 2000). This interaction was hypothesized to reduce the likelihood of Ca^{2+} channel opening at synaptic vesicle docking sites that were unoccupied, thus preventing Ca^{2+} entry into the neuron at sites where there was no need for such entry. This functional modulation of Ca^{2+} channels by syntaxin appeared to depend on a couple amino acid residues in syntaxin, that when mutated, abolished channel modulation without affecting binding (Bezprozvanny et al., 2000). The dual role for syntaxin-Ca^{2+} channel interactions in both co-localizing Ca^{2+} channels with the vesicle release apparatus and modulating Ca^{2+} channel function was supported by physiological studies at neuromuscular synapses using a combination of synprint peptides and mutated syntaxin proteins (Keith et al., 2007). Thus, Ca^{2+} channel interactions with SNARE proteins are hypothesized to be important regulators of fast, synchronous transmitter release. These interactions are thought to co-localize Ca^{2+} channels with synaptic vesicle release sites and may control Ca^{2+} channel function such that channel gating is influenced by vesicle docking.

However, this synprint site on Ca^{2+} channels may not be sufficient to regulate Ca^{2+} channel targeting to the active zone. Some studies have found that exchange of the synprint site from a Cav2.1 (P/Q-type) channel into a Cav1.2 (L-type) channel could create a synapse between superior cervical ganglion neurons in which transmitter release was controlled by these "hybrid" Cav1.2 (L-type) channels (Mochida et al., 2003). However, when Szabo et al. (2006) inserted the synprint site into a Cav1.2 channel (which normally does not traffic to the nerve terminal), this simple addition could not cause this channel to traffic to nerve terminals of hippocampal neurons. Therefore, there are likely to be other interactions that are critical for the trafficking and active zone localization of Ca^{2+} channels (see below).

Furthermore, the synprint site is absent from presynaptic Ca^{2+} channels expressed at most invertebrate synapses (Littleton and Ganetzky, 2000). In these cases, there may be other regions of the channel that serve to aid in binding to the synaptic vesicle release site. Specifically, in Lymnaea neurons, presynaptic Ca^{2+} channels have an alternatively spliced C-terminus that interacts with active zone scaffold proteins to anchor them near transmitter release sites (Spafford et al., 2003). This mechanism of C-terminus anchoring may be shared by mammalian Cav 2.3 Ca^{2+} channels when they are present at synapses (Table 1), although these channels are often not present in the active zone region of synapses, and can engage in other peri-active synaptic roles (Dietrich et al., 2003).

1.1.5. Auxiliary subunits of the Ca^{2+} *channel*

As described above, voltage-gated Ca^{2+} channels are assembled as multimeric proteins with a pore forming α1 subunit and auxiliary β and α2δ subunits (figure 1). These channels appear to require the auxiliary β and α2δ subunits for effective trafficking of the α1 subunit out of the Golgi apparatus and into the cell membrane (Pragnell et al., 1994; Canti et al., 2005; Davies et al., 2006; Hendrich et al., 2008; Dolphin, 2003; 2013). In fact, the α2δ subunit in particular has been implicated in a variety of signaling roles (Dolphin, 2013). With respect to Ca^{2+} channel

positioning within the nerve terminal active zone, Hoppa and colleagues (2012) have provided evidence for two distinct effects. First, they showed that over-expression of α2δ subunits in hippocampal neurons caused an increase not only in the presynaptic concentration of α2δ protein, but also in the total concentration of the endogenous presynaptic Ca^{2+} channel α1 subunit. This seems to be a trafficking role that brings Ca^{2+} channels to the nerve terminal. Second, they provided evidence that α2δ subunits signal with an as yet unidentified extracellular protein in the synaptic cleft via their extracellular metal ion-dependent adhesion site (MIDAS). This signaling led to an apparent increase in the tightness of the spatial coupling between active zone Ca^{2+} channels and synaptic vesicles (figure 1). Overexpression of the α2δ Ca^{2+} channel subunit led to an increase in the probability of transmitter release and a decrease in the sensitivity to disruption by Ca^{2+} chelators (Hoppa et al., 2012). Thus it was hypothesized that α2δ subunits increased Ca^{2+} channel density within the active zone and increased coupling of Ca^{2+} channels to docked synaptic vesicles. Since the extracellular synaptic cleft interaction of the α2δ subunits via MIDAS may be dependent on divalent cations, it is interesting to note that a 3-hour exposure of the frog neuromuscular junction to zero extracellular Ca^{2+} disrupts active zone protein organization (presumably including the presynaptic Ca^{2+} channels), resulting in an increase in paired pulse facilitation that may be a consequence of a loosening of the coupling between Ca^{2+} channels and docked synaptic vesicles (Meriney et al., 1996). A more complete understanding of these interactions and their consequences will require further experimentation.

1.2. The activation of presynaptic Ca^{2+} channels by action potentials

Despite the variety of mechanisms discussed above that work to bring presynaptic active zone Ca^{2+} channels in close proximity to docked synaptic vesicles, during action potential invasion of the nerve terminal, at many synapses there appears to be a low probability that Ca^{2+} channels will open to gate the Ca^{2+} influx that triggers vesicle fusion (Pumplin et al., 1981; Llinas et al., 1982; King and Meriney, 2005; Yang and Wang, 2006; Tarr et al., 2013). This can occur

for several reasons. First, in general, voltage-gated Ca^{2+} channels are relatively slow to open. All voltage-gated channels sense voltage using the charged amino acids within the S4 segments of the four domains that make up the pore-forming α1 subunit. Sodium channels can accomplish voltage-dependent gating by S4-mediated conformational changes very quickly (Lacroix et al., 2013), but Ca^{2+} and potassium channels are generally slower to respond to voltage depolarization. Often, Ca^{2+} channels have not been reported to begin to open until the repolarization phase of the action potential (although see discussion below when studied at higher temperature), at which point sodium channels have already opened and inactivated (Borst and Sakmann, 1998; Pattillo et al., 1999).

The relatively slow response by Ca^{2+} channels to voltage depolarization is only one of many issues that control channel gating during an action potential. As such, there is some variability in the reported effectiveness of the action potential waveform at activating Ca^{2+} channel opening (Borst and Sakmann, 1998; Bischofberger et al., 2002; King and Meriney, 2005; Luo et al., 2011). This variability in Ca^{2+} channel activation by action potentials likely results from variability in a few important conditions, which have a significant impact of Ca^{2+} channel gating. First, the subtype of Ca^{2+} channel can have a significant impact on the kinetics of activation, with some channels responding faster than others (Kasai and Neher, 1992; King and Meriney, 2005); although as gauged by kinetics of gating currents, the types commonly present at synapses (Cav2.2 and Cav2.1; N- and P/Q types respectively) appear to have similar activation kinetics (Barrett et al., 2005; Jones et al., 1997; 1999). Second, the shape of the action potential waveform can vary among synapses, and is also very important. At some synapses these action potentials have relatively long durations (more than one msec duration at half amplitude), while at other synapses the action potential is very short (~200 μsec). In fact, at some synapses, the action potential waveform changes duration over the course of developmental maturation (Taschenberger and von Gersdorff, 2000; Fedchyshyn and Wang, 2005; Yang and Wang, 2006). At the immature calyx of Held synapse, the action potential is

relatively broad and this waveform activates the majority of synaptic Ca^{2+} channels (Borst and Sakmann, 1998; Taschenberger and von Gersdorff, 2000; Fedchyshyn and Wang, 2005). However, as the calyx of Held synapse matures, the action potential waveform becomes much briefer, and concomitantly is less effective at activating presynaptic Ca^{2+} channels (Taschenberger and von Gersdorff, 2000; Fedchyshyn and Wang, 2005). Furthermore, the amplitude of the action potential waveform is also a critical variable in Ca^{2+} channel activation because a stronger action potential depolarization activates a greater fraction of presynaptic Ca^{2+} channels. Action potentials that rise to more depolarized potentials will open more Ca^{2+} channels both because of the greater fraction of Ca^{2+} channel activation that occurs with stronger depolarizations, and because channel activation kinetics are voltage-dependent and faster at more depolarized potentials (Kay and Wong, 1987; Jones and Marks, 1989). Recordings from chick ciliary ganglion neurons and rat calyx of Held synapses have shown, for example, that the activation time constant for Ca^{2+} channels is significantly slower at -10 mV (0.8-0.9 msec) than at +30 mV (0.12-0.3 msec; Borst and Sakmann, 1998; Pattillo et al., 1999). Ca^{2+} channel activation kinetics are also very sensitive to temperature (Nobile et al., 1990; McAllister-Williams and Kelly, 1995). As temperature was varied in recordings from parallel fiber synapses in the cerebellum, the temperature-sensitive changes in many of the above mentioned factors led to significant changes not only in the action potential waveform, but also in the Ca^{2+} current activation kinetics (Sabatini and Regehr, 1996; 1997). In fact, when these synapses were studied at warm physiological temperatures, Ca^{2+} channels gated quickly enough to open during the upstroke of the action potential (in contrast to the generalizations presented above).

Overall, Ca^{2+} channel activation kinetics (which vary with voltage and temperature) combine with action potential waveform shape to govern the likelihood that presynaptic Ca^{2+} channels will open within the transmitter release site.

1.3. Functional organization of presynaptic single vesicle release sites

The number of Ca^{2+} channels in close proximity to each docked synaptic vesicle, and the probability that Ca^{2+} channels open during an action potential, have a strong impact on the function of transmitter release sites. Different synapses appear to express different numbers of voltage-gated Ca^{2+} channels in active zone regions of the nerve terminal. These differences in expression may be governed by active zone size, and the number of "slots" available for Ca^{2+} channel insertion within the active zone. Further, the spatial coupling of active zone Ca^{2+} channels to the synaptic vesicle release site is likely to be regulated by the protein-protein interactions discussed above and outlined in figure 1.

When very few Ca^{2+} channels open during an action potential in each active zone, and these channels are tightly coupled to the synaptic vesicle release site, the flux through single open Ca^{2+} channels (often termed a local "nanodomain" of spatial Ca^{2+} rise near the mouth of a single open channel) normally triggers synaptic vesicle release (figure 2; Eggermann et al., 2012; Tarr et al., 2013). This "nanodomain" coupling is often unreliable for two reasons. First, if there are only a few Ca^{2+} channels associated with each synaptic vesicle, the probability that an action potential waveform will open one of these channels is likely to be low. Second, even when a closely associated Ca^{2+} channel does open and allow presynaptic Ca^{2+} influx, the likelihood that this Ca^{2+} flux triggers vesicle fusion for the closely associated synaptic vesicle can also be low (Luo et al., submitted). As such, these types of single vesicle release sites are often unreliable (Tarr et al., 2013).

At synapses where there are many Ca^{2+} channels within the active zone, there is often thought to be variable spatial coupling of these channels to any individual single vesicle release site. Under these conditions, the flux through many Ca^{2+} channels (termed a "microdomain" of spatial Ca^{2+} rise that is summed from all open channels) often triggers each synaptic vesicle fusion event (figure 2). This "microdomain" coupling may be more reliable because there are so many Ca^{2+} channels in the active zone that even if they all don't open during each stimulus, the

total action potential-evoked Ca^{2+} influx is robust and of similar magnitude during each stimulus. As such, there is usually a reliable release of synaptic vesicles at these synapses because many docked synaptic vesicles are exposed to a relatively uniform Ca^{2+} signal during each stimulus (Tarr et al., 2013).

Interestingly, with developmental maturation, synapses may convert from microdomain to nanodomain coupling of Ca^{2+} channels to the transmitter release apparatus (Taschenberger and von Gersdorff, 2000; Fedchyshyn and Wang, 2005). This is perhaps most well studied at the calyx of Held synapse where immature synapses use relatively broad action potentials to activate a large fraction of available Ca^{2+} channels and trigger transmitter release using the summed Ca^{2+} entry from many open channels. However, as these synapses mature, the action potential narrows significantly, and it appears that synaptic vesicle release is increasingly triggered by the Ca^{2+} flux through fewer open Ca^{2+} channels that are likely more closely associated with individual docked synaptic vesicles (Taschenberger and von Gersdorff, 2000; Fedchyshyn and Wang, 2005).

At many adult synapses, it may be the case that nanodomain coupling between Ca^{2+} influx and vesicle fusion is predominant. In any case, the speed and reliability of Ca^{2+}-triggered synaptic vesicle fusion is controlled by the events described above that serve to position individual Ca^{2+} channels very close to docked synaptic vesicles, and the action potential depolarization conditions that gate these channels.

2. Molecular machinery of fast exocytosis

Most current models invoke interactions among four proteins, synaptotagmin I (or II), synaptobrevin 2, syntaxin 1A/B, and SNAP-25 as being crucial for driving the fusion of the synaptic vesicle membrane with the plasma membrane. The following discussion traces the

origins of these models and addresses gaps in our understanding of how these interactions contribute to the rapid, synchronous release of neurotransmitter.

2.1 Molecular Mechanisms Underlying the Ca^{2+}-dependent, Synchronous Release of Neurotransmitter from Synaptic Vesicles

The preceding section surveyed the role of presynaptic Ca^{2+} channels in mediating and regulating Ca^{2+} entry into nerve endings. Once Ca^{2+} ions enter a nerve terminal, they interact with one or more molecular targets to trigger the rapid, synchronous release of neurotransmitter from synaptic vesicles (Katz, 1966, 1969). A major focus of this section is to review the efforts to identify the protein or proteins that serve as Ca^{2+} sensors for the fast, stimulus-dependent release of transmitter at nerve endings. Although several mechanistic questions remain to be answered, it is almost universally accepted that members of the p65/synaptotagmin family of synaptic vesicle proteins fulfill the role of Ca^{2+} sensors for the fast, synchronous release of transmitter at most nervous system synapses. If synaptotagmins are the exocytotic Ca^{2+} sensors, how do they work? The consensus that emerged over the last 20 years points to a likely interaction of synaptotagmin with a trio of proteins known as SNAREs (soluble, N-ethylmaleimide-sensitive factor attachment protein receptors). However, in spite of immense effort, little agreement has been reached concerning the mechanism by which synaptotagmin interfaces with SNAREs to promote synaptic vesicle exocytosis. Currently, at least six different models remain under consideration (Jahn and Fasshauer, 2012). A central thesis of the present review is that the synaptotagmin-SNARE debate is a red herring. Instead, a hypothesis was recently advanced in which SNAREs bring vesicles close to plasma membrane, but Ca^{2+} binding to synaptotagmin can catalyze the actual rapid exocytosis event at nerve terminals without direct involvement of SNAREs (Gundersen and Umbach, 2013). The advantages of this “synaptotagmin-only” model for the final fusion step will be discussed after a systematic critique of the shortcomings of SNARE-based models of fast exocytosis:

SNAREs were discovered in a biochemical screen designed to capture proteins conferring specificity to Golgi membrane trafficking (Sollner et al., 1993a). The SNARE proteins included synaptobrevin 2 (also known as VAMP 2 for vesicle-associated membrane protein 2) a previously reported constituent of synaptic vesicles (Trimble et al., 1988; Elferink et al., 1989; Baumert et al., 1989), along with SNAP-25 and syntaxin. SNAP-25 (synaptosome-associated protein of 25 kDa) had been identified as a synaptosomal membrane protein (Oyler et al., 1989), while syntaxin was a synaptic plasma-membrane protein that interacted with synaptotagmin (Bennett et al., 1992). In conjunction with data indicating that the SNAREs were targets of the clostridial neurotoxins, it soon became apparent that SNAREs might do more than mediate the specificity of intracellular membrane trafficking. Indeed, evidence that SNARE proteins formed a heterotrimeric complex led to a proposal for an expanded role for SNAREs in membrane fusion (Sollner et al., 1993b). Subsequently, SNARE-based hypotheses (Fig.3) have dominated the discussion of intracellular membrane fusion. At this point, it is important to note that several other SNARE-interacting proteins are likely to contribute to the regulation of SNARE-mediated cellular events. These proteins include unc/munc-13, unc/ munc-18 and complexin. In general, this review will not address the function of these proteins, because excellent discussions have appeared (Brose and Rosenmund, 2002; Weimer and Jorgensen, 2003; Toonen and Verhage, 2007; Sudhof and Rothman, 2009; Sorensen, 2009; Jahn and Fasshauer, 2012; Barclay et al., 2012). Instead, the primary focus of the ensuing presentation concerns the proposed mechanisms (Fig.3) by which SNAREs promote the final stage of membrane fusion, and ideas for how synaptotagmin might regulate this process.

How do SNAREs work? The core idea took root in the finding that SNAREs tended to be distributed in separate membrane compartments. Synaptobrevin family members (v-SNAREs or vesicular SNAREs) were typically present on synaptic vesicles or other secretory organelles, while SNAP-25 and syntaxin (t-SNAREs, or target SNAREs) resided on the plasma membrane. Consequently, it was surmised that when a synaptic vesicle approached sufficiently closely to

the plasma membrane, its v-SNAREs could bind to t-SNAREs. In other words, synaptobrevins could tether the vesicle to the plasma membrane by interacting in “trans” with t-SNARES (Fig.3). Structurally, this interaction exhibited a 1:1:1 stoichiometry (Fasshauer et al., 1997) and was found to involve α-helical regions of each of these proteins. Moreover, the process of intermolecular coiling proceeded in “zipperlike” fashion from the amino (N)-terminus of each SNARE’s helical region toward the carboxyl (C) terminal membrane spanning domains of syntaxin and synaptobrevin (Fig.3 and Hanson et al., 1997; Sutton et al., 1998; Poirer et al., 1998; Weber et al., 1998). Importantly, the two, α-helical regions of SNAP-25, which is tethered to the plasma membrane by fatty acyl moieties, also coiled from N to C (Fig.3). Along with the large free energy change associated with this reaction (Fasshauer et al., 1997), it became intuitively apparent that the parallel zippering of SNAREs shown in Fig.3 should facilitate the tight (or, tighter) apposition of the vesicular and plasma membranes. Via mechanisms that will be discussed later, this close apposition of membranes was presumed to lead to fusion. The exception to this general idea is model D (Fig. 1). Here, SNAREs form part of the fusion pore which is thought to dilate as a consequence of SNARE coiling (Jackson, 2010).

The proposals in Fig.3 are based on convergent input from a substantial body of research featuring anatomical, biochemical, biophysical and genetic approaches. Hence, it is important to review the advantages and disadvantages of the different empirical strategies that have contributed to these models (see text box below). As a prelude to this discussion, it is necessary to consider two sets of observations which significantly constrain the mechanisms underlying synchronous transmitter release at nerve endings. The initial observations concern the kinetics of nerve terminal exocytosis. The second set of observations emerged from studies of membrane fusion using protein-free liposomes.

2.1.1 *Kinetics of exocytosis at nerve terminals*:

Regulated exocytosis appears to be present in most, if not all eukaryotic cells (Andrews, 2000; Han and Campbell, 2007). However, nerve terminals are distinctive in that the fusion of synaptic vesicles with the plasma membrane occurs exceedingly rapidly. This conclusion evolved from measurements originally made by Kuffler (1942) and more systematically by Katz and Miledi (1965), who observed that the delay between presynaptic activation and a post-synaptic response was ~1 msec at frog neuromuscular junctions. Of course, this 1 msec delay included many steps in addition to the exocytotic event itself. Further work by Llinas and colleagues (1981) using the squid giant synapse showed that the time course of presynaptic Ca^{2+} entry accounted for a large fraction of the synaptic delay. Overall, they concluded that Ca^{2+} sensing and the exocytotic event itself contributed only about 0.2 msec to the synaptic delay. Additional insight into this situation came when Sabatini and Regehr (1996) applied a combination of imaging and electrophysiological techniques to demonstrate that synaptic vesicles in rat brain can undergo exocytosis within 60 μs of Ca^{2+} entry into the nerve terminal. Taken together, these results have very important implications. Specifically, they indicate that whatever molecular machinery is used by nerve terminals to sense Ca^{2+} entry and to convert the Ca^{2+} signal into the exocytotic fusion between the synaptic vesicle membrane and the plasma membrane has to operate incredibly quickly. What is meant by “incredibly quickly”?

Among other things, “incredibly quickly” strongly suggests that it is only members of the population of synaptic vesicles that are very close to (~1 nm) or in direct contact with the nerve terminal plasma membrane that can undergo exocytosis. The reasoning behind this assertion is that 60 μsec almost certainly provides insufficient time to recruit vesicles that are >1-2 nm from the plasma membrane. This constraint was first addressed theoretically by Parsegian [as recounted by Llinas and Heuser,1977]. He calculated that if synaptic vesicles were viewed as Brownian particles in a milieu with a viscosity of 10-100 poise, these vesicles could move 0.5-5 nm during a 0.2 msec time window (relevant to exocytotic events in squid). Clearly, this distance is appreciably less for secretory events occurring within 60 μsec. However, this calculation

ignores any other reactions that must occur during the triggering of membrane fusion, such as Ca^{2+} sensing or formation of a fusion pore, and renders it theoretically implausible that vesicle movements exceeding 1-2 nm occur as a prelude to nerve terminal exocytosis. Further support for this conclusion comes from studies which monitored the rate of synaptic vesicle translocation at ribbon synapses. Measurements made using total internal reflection fluorescence (TIRF) microscopy indicated that synaptic vesicles could approach the plasma membrane at speeds of ~800 nm-sec^{-1} (Zenisek et al., 2000; Chen et al., 2013). This rate of vesicle translocation is clearly inadequate to move a synaptic vesicle even a fraction of one nm within 60 μsec. Nevertheless, a recent hypothesis has invoked nm scale movements of synaptic vesicles as a mechanism to engage SNARE proteins for the initiation of exocytosis (van den Boogart et al., 2011; Jahn and Fasshauer, 2012). Clearly, for this hypothesis to be tenable, it will be necessary to demonstrate that synaptic vesicles can translocate much faster than theory or practice currently indicate that they are capable of moving.

"Incredibly quickly" also strongly suggests that any macromolecular constituents of the exocytotic machinery must be suitably pre-positioned to respond to the Ca^{2+} signal and to execute their downstream reactions. In other words, there is insufficient time to recruit proteins besides those that are situated at or very near the site of exocytosis. Formally, this argument implies that as the electron microscopic localization of presynaptic proteins continues to improve, it should become possible to exclude candidate participants in the membrane fusion reaction, because their sub-cellular location is inappropriate. At the same time, it also means that it will be extremely challenging to study dynamic steps in the exocytotic reaction sequence. For instance, it was calculated that even when using a liquid helium-based cryofixation procedure, it took ~1-2 msec to freeze frog motor nerve terminals for ultrastructural analysis (Heuser et al., 1979). Moreover, in spite of the introduction of protocols such as high pressure cryofixation which minimize ice crystal formation (Marti et al., 1987; Dubochet, 1995; Rostaing et al., 2006; Weimer et al., 2006), it will remain a challenge to trap events that occur in <100

μsec. Finally, although amazing progress has been made in super-resolution microscopy (Betzig et al., 2006; Hell, 2007), the rate of image capture currently is orders of magnitude slower than is needed to follow secretory events at nerve terminals (van de Linde et al., 2012). Consequently, the dynamics of synaptic vesicle exocytosis remains an area of research that will benefit from the development of imaging technology that is capable of detecting events operating on nm scales and in the μsec time domain.

2.1.2 *Insights into the fusion of biological membranes derived from the use of model membrane systems:*

As evidence for the biological importance of membrane fusion accumulated (Lucy, 1970; Poste and Allison, 1973), several groups sought to recapitulate this process *in vitro* by using vesicles prepared from lipids that are typically present in biological membranes. Although early phases of this work were subjected to suitably harsh criticism (by Gingell and Ginsberg, 1978, who questioned the interpretation of the "fusion" assays), ongoing efforts culminated in the development of methods to form lipid bilayer vesicles (liposomes) *in vitro*; to distinguish membrane fusion from either aggregation or lysis; and to understand the impact on membrane fusion of a host of variables, including the lipid composition of the vesicles, the diameter of the liposomes, temperature, osmotic pressure, salt, divalent cations and other fusion-promoting agents (Maeda and Ohnishi, 1974; Prestegard and Fellmeth, 1974; Papahadjopoulos et al., 1974,1976a,b; 1977; Kantor and Prestegard, 1975; Lau and Chan, 1975; Breisblatt and Ohki, 1975, 1976; Dunham et al., 1977; Koter et al., 1978; Olson et al., 1979; Portis et al., 1979; Verkleij et al., 1979; 1980; Liao and Prestegard, 1979a,b, Wilschut and Papahadjopoulos; 1979; Duzgunes et al., 1981a,b; 1987; Sundler et al., 1981; Wilschut et al., 1980, 1981; Bearer et al., 1982; Ohki, 1982, 1984). Complementing these studies were experiments monitoring the fusion of liposomes with planar lipid bilayers, a system which topologically better mimics the biological situation (Cohen et al., 1980, 1984; Zimmerberg et al., 1980a,b; Duzgunes and Ohki, 1981;

Perin and McDonald, 1989). An important conclusion that emerged from these studies is that membranes composed of biologically relevant anionic lipids can fuse independently of protein. In other words, fusion is a process that is intrinsic to biological membrane lipids and does not require protein. Nevertheless, in the same fashion that enzymes lower the activation energy for chemical reactions, it has been widely inferred that proteins (as well as a large number of chemical fusogens), lower the energy barrier for membrane fusion in cells. Additional implications of this *in vitro* work for biological membrane fusion were addressed in early reviews (Rand and Parsegian, 1986; Papahadjopoulos et al., 1990), and led to the following framework for understanding this process:

Empirical investigations of liposome fusion spawned hypotheses that sought to explain the molecular transitions that must occur at the interface between two lipid bilayers to achieve membrane fusion (Gingell and Ginsberg, 1978). Although various different fusion scenarios have been suggested, the proposals receiving the most attention involve either an inverted micelle intermediate (Lau and Chan, 1975; Verkleij et al., 1980; Ohki, 1982), or the transient formation of a lipid stalk which proceeds via a hemi-fusion intermediate to full fusion (Gingell and Ginsberg, 1978; Hui et al., 1981; Markin et al., 1984; Kozlov et al., 1989; Kozlovsky et al., 2002). The ensuing evolution of these ideas included a systematic consideration of the relative energetics of the inverted micelle and stalk models which led to arguments in favor of a stalk intermediate (Siegel, 1993; 1999; Malinin and Lentz, 2004), as well as empirical data arguing against the inverted micelle model (Bearer et al., 1982) and in favor of stalks (Yang and Huang, 2002). More recently, the preponderance of evidence has pointed to the idea that lipid bilayer membranes proceed via stalk and hemi-fusion intermediates on the path to full membrane fusion (Lee and Lentz, 1997; Markin and Albanesi, 2002; Kozlovsky and Kozlov, 2002; Kozlovsky et al., 2002; Siegel, 2008). Interestingly, in an investigation of ligand- or electric field-induced fusion of large, unilamellar vesicles, it was observed that fusion necks developed with sub-millisecond kinetics and expanded at rates exceeding 1 cm-sec^{-1} (Haluska et al., 2006). It

remains to be determined whether these fusion events evolved via stalk or hemi-fusion intermediates, and whether a similar fusion pathway is applicable to smaller diameter liposomes. Nevertheless, the global relevance of these model systems to the exocytotic fusion between synaptic vesicles and the plasma membrane is the assumption that the proteins mediating events at nerve terminals exploit a fusion pathway that is similar to what occurs with liposomes, *in vitro*. Indeed, a theme that is evident in Fig. 3 and will re-emerge in later discussions is that many models of nerve terminal exocytosis invoke the formation of a stalk or hemi-fusion intermediate as a prelude to biological membrane fusion. In this context, it is interesting that evidence for synaptic vesicle hemi-fusion was reported for rat central synapses (Zampighi et al., 2006), but additional work will be needed to confirm this pathway.

2.2 Empirical strategies to characterize proteins of the nerve terminal fusion apparatus

*****It is the authors' intention to have the entire following section (2.2) set in a text box with slightly smaller font (here, 10 versus 11) (or perhaps better as on online supplement) so that experts in the field can skip this section, if desired*****

Over the last three decades, a variety of experimental approaches has contributed to the identification of proteins that are now recognized as being crucial for exocytosis at nerve terminals. Because no single experimental strategy is adequate to provide a definitive explanation of the function of these proteins, the field has relied on results from several different disciplines. Thus, the remainder of this section reviews the advantages and limitations of the most commonly used approaches for identifying and studying "exocytotic" proteins at nerve terminals. The goal of this narrative is not to undertake an exhaustive, technical review of each empirical approach. Rather, the purpose is to alert the reader to issues of data interpretation that are particularly important when considering events that take place at structures as small as nerve terminals and as rapidly as synaptic vesicle exocytosis.

2.2.1 *Light and Electron Microscopy:*

Obviously, for a protein to play a role in synaptic vesicle exocytosis, it has to be present at nerve terminals. As noted below, a variety of light and electron microscopic techniques can serve as part of this

assessment. In addition, a rapidly expanding array of imaging approaches holds considerable promise for helping to clarify the mechanism of exocytosis at nerve terminals. Selected examples will be outlined below.

At the light microscope level, the presence of exocytotic proteins at nerve endings can be detected by direct or indirect immunofluorescence microscopy, as well as by the addition to the protein of genetically encoded tags, such as variants of green fluorescent protein (GFP). A general boon to research in this area has been the fact that well-characterized primary antibodies are now available from a number of commercial sources, and some publications (like, The Journal of Comparative Neurology) provide resources to help investigators identify suitable antibodies (this web link is to the antibody resource: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1096-9861/homepage/jcn_antibody_database.htm.) Consequently, light microscope immunohistochemistry can serve as a good, global index of the relative level of expression of native or altered proteins. Nevertheless, it is still necessary to bear in mind the importance of control experiments (for a good discussion of controls, see Saper, 2005) for the reliable immunohistochemical localization of a protein, as well as the resolution limits that persist even in this era of super-resolution light microscopy (van de Linde et al., 2012).

As a complementary strategy to antibody experiments, the addition to a protein of a genetically encoded fluorescent tag has helped to resolve questions concerning the subcellular distribution of various proteins. For instance, a recent survey of the compartmental targeting of many of the 17 different mammalian synaptotagmin gene products concluded that only synaptotagmins I and II were present at nerve endings while the rest of the synaptotagmins localized in axons or dendrites of hippocampal neurons, *in vitro* (Dean et al., 2012). However, in addition to issues of detection limits, one needs to take care that the presence of a genetically encoded tag does not alter the distribution of the parent protein. A common control for this latter concern is to compare the localization of variants with the fluorescent tag inserted at different sites on the same protein (for instance, at the N versus C terminus). Nevertheless, as a final admonition, if one is concerned about the distribution of a protein on the nm scale that is relevant to nerve terminal exocytosis, electron microscopy remains the preferred option.

In addition to static images of protein distribution, fluorescence microscopy has made valuable contributions to the understanding of dynamic events at nerve terminals. It was noted earlier that fluorophores have been exploited to detect and quantify changes in cytosolic Ca^{2+} ion activity at nerve endings, and Ca^{2+} dyes can be used as an indirect gauge of secretory activity. In addition, two other fluorescence imaging strategies have been widely employed. The first uses dyes of the "FM" family (such as, FM1-43 and FM4-64; for review, see Rizzoli et al., 2003; Alabi and Tsien, 2012; Hoopmann et al., 2012) which become trapped in recycling synaptic vesicles. Nerve terminals that have incorporated FM dyes into the lumen of synaptic vesicles are typically monitored through subsequent rounds of activity during which dye unloading occurs. This approach has been used in conjunction with TIRF imaging to detect synaptic vesicle discharge at ribbon synapses (Zenisek et al., 2000; Holt et al., 2004; Chen et al., 2013), and in synaptosomes (Serulle et al., 2007). FM dyes are also particularly advantageous for studying secretory dynamics in systems that are not readily accessible to electrophysiological analysis and have been especially useful for advancing our knowledge of synaptic vesicle recycling (Smith et al., 2008; Dittman and Ryan, 2009). However, one caveat when using the FM dyes is that they can have subtle effects on the probability of vesicle fusion (Zhu and Stevens, 2008). The second dynamic imaging approach uses pH-sensitive variants of GFP which are coupled to the luminal domain of synaptic vesicle proteins (Miesenbock et al., 1998). Although these so-called pHluorin molecules were originally touted as having a range of prospective uses (Ashby et al., 2004), it appears that their greatest utility lies in the optical assessment of circuit dynamics (Dreosti and Lagnado, 2011), rather than studying secretion at individual nerve terminals. However, as noted above, pHluorin tags were recently utilized to investigate the subcellular distribution and recycling of synaptotagmin isoforms (Dean et al., 2012), so they clearly have utility in this context. Finally, a fluorescence resonance energy transfer (FRET)-based approach to monitor SNARE interactions was recently applied to hippocampal nerve terminals (Degtyar et al., 2013), but the temporal resolution of this technique was inadequate to give insight into molecular events that occur during synchronous transmitter release.

Electron microscopy has played a crucial role in the evolution of ideas concerning chemical synaptic transmission. For instance, it provided the final proof of the cytoplasmic discontinuity of pre- and post-synaptic elements, and it supplied the first indication of the existence of synaptic vesicles. Later, freeze

fracture images of frog motor nerve terminals undergoing massive exocytosis lent morphological credence to the vesicle hypothesis (Heuser, 1976; Heuser et al., 1979; Heuser and Reese, 1981), and led to proposals concerning the identity of Ca^{2+} channels in the nerve terminal plasma membrane (Heuser, 1976; Llinas and Heuser, 1977; Pumplin et al., 1981). However, it is only recently that further progress has been made in achieving one of the long-standing goals of electron microscopy: namely, the development of procedures for identifying individual macromolecules at synapses. Such efforts have abundant potential for helping to distinguish among different models of synaptic vesicle exocytosis, so the next several paragraphs will highlight where technical advances may be particularly beneficial.

Freeze-fracture images of frog motor nerve terminals have long tantalized investigators with the regular spacing of large particles (presumably, representing integral membrane proteins, including presynaptic Ca^{2+} channels) at active zones and the ability to capture profiles of what almost certainly are synaptic vesicle exocytosis and endocytosis (Dreyer et al., 1973; Peper et al., 1974; Ceccarelli et al., 1979; Heuser et al., 1979; Heuser and Reese, 1981; Miller and Heuser, 1984; Pawson et al., 1998). However, there has been meager progress in assigning protein identities to individual particles in freeze-fracture replicas. While there are good reasons to accept the original arguments concerning the identification of presynaptic Ca^{2+} channels at frog motor nerve terminals (Pumplin et al., 1981), virtually no information has been gleaned from this technique for the other proteins of the exocytotic cascade. In spite of progress in protein localization via transmission electron microscopy, now may be a propitious time to re-visit this issue. For instance, the investment of efforts to develop genetically encoded tags to identify proteins in freeze-fracture replicas could potentially pay handsome dividends in validating the localization of presynaptic Ca^{2+} channels or t-SNAREs. Alternatively, a more-robust protocol for antibody labeling of freeze-fracture particles would aid the particle-identification problem (Severs and Robenek, 2008). Or, the development of strategies to improve the detection of small, integral membrane proteins, like, the synaptobrevins or synaptotagmins, which have single membrane-spanning domains, would also advance this field. For instance, there is considerable disagreement in the estimated stoichiometry of such synaptic vesicle constituents, as SV2, synaptobrevin 2 and synaptotagmin 1. Takamori and colleagues (2006) reported an average of ~2 SV2 proteins, 70 synaptobrevin 2 molecules and 15 synaptotagmins per vesicle, while the comparable figures from Mutch and co-workers (2011) were 5, ~11 and 7,

respectively. By the same token, the number of particles (which presumably represent integral membrane proteins) per vesicle detected in freeze fracture replicas of frog nerve terminals is appreciably lower than would be expected from either of these biochemical estimates (Heuser and Reese, 1981). Clearly, under suitable detection conditions, freeze-fracture analysis has the potential to provide an independent assessment of the number of integral membrane proteins in a “typical” synaptic vesicle. At the same time, because it can expose large patches of the presynaptic plasma membrane, freeze fracture could help clarify the density (number per unit area) and localization of SNAREs in the presynaptic active zone. In this context, later discussions will identify circumstances in which high-resolution protein localization data will be extremely beneficial in helping to distinguish among different theories of synaptic vesicle exocytosis.

An approach that has shown increasing promise in identifying molecular components of the exocytotic machinery is high-resolution (2-3 nm) tomographic reconstruction of active zones coupled with the analysis of protein complexes associated with synaptic vesicles and/or the active zone material (Harlow et al., 2001; Nagwaney et al., 2009; Szule, et al., 2012; Harlow et al., 2013; see also Fernandez-Busnadiego et al., 2013). Much of this work has taken advantage of the stereotypical structure of active zones at frog neuromuscular junctions, and it has led to the identification of a number of distinctive assemblies which are organized in a reproducible pattern. Although plausible suggestions have been made regarding the identity of the protein constituents of these assemblies (Szule et al., 2012; Harlow et al., 2013), it will require additional work to verify these assignments. Clearly, along with freeze-fracture analyses, this approach holds abundant promise for advancing our understanding of molecular components of the exocytotic machinery at nerve terminals.

The use of antibodies to identify proteins in transmission electron micrographs has been employed effectively by many groups (Harris and Weinberg, 2012). However, this approach can be problematic, because the harsh fixation conditions normally required for adequate structural preservation are often incompatible with good antibody labeling. Nevertheless, with appropriate care, this technique can provide a powerful adjunct to other strategies for high-resolution mapping of a protein’s distribution (Mayhew, 2011) and refinements of this approach continue to be made (Jiao et al., 2010). Recently, alternatives to antibody labeling have been developed which use genetically encoded tags to correlate light and electron

microscopic data (Shu et al., 2011; Watanabe et al., 2011). In an application of this technique, interesting insights emerged into the subcellular localization of α-synuclein at nerve endings (Boassa et al., 2013), and further efforts along these lines harbor obvious potential for illuminating the distribution of other synaptic proteins.

Even if it proves to be technically difficult to identify and resolve SNAREs at the nm level, electron microscopic approaches retain considerable potential to help distinguish among the different SNARE models of Fig.3. For instance, the vast majority of electron microscopic images of nerve terminals reveal a small population of synaptic vesicles which appear to be in direct contact with the presynaptic plasma membrane. However, most of these images were obtained by using conventional chemical fixatives which might artificially influence the area of contact between the vesicle and plasma membrane. In other words, we currently lack sufficient information to distinguish the annulus version (Fig.3A, direct contact between the vesicle and plasma membrane) from the nipple theory (Fig.3B, which requires a 1-3 nm separation of the vesicle and plasma membrane) or the "hover" variant (Fig.3C in which the vesicle presumably is >4 nm from the plasma membrane). This issue will be discussed in greater detail below.

In summary, technological advances have put us on the verge of major improvements in understanding the ultrastructural features and dynamics of nerve terminals that are relevant to synaptic vesicle exocytosis. The coming decade should shed even more light on this issue.

2.2.2 *Biophysical Analyses:*

A major contribution to our understanding of the molecular mechanism of synaptic vesicle exocytosis has come from the analysis of biophysical data obtained using preparations subjected to genetic or other perturbation strategies. Setting the stage for these efforts were the classical studies of neuromuscular transmission which used intracellular microelectrodes to assess the properties of spontaneous and stimulus-evoked transmitter release (Katz, 1969). Subsequently, patch clamp techniques (Hamill et al., 1981) dramatically expanded the range of preparations that were amenable to biophysical studies of synaptic function. This reliance on electrophysiological techniques reflects the high temporal resolution as well as the exquisite sensitivity that can be achieved. Among the compelling examples of the versatility of these approaches are the estimate of the number of acetylcholine molecules in a quantum (Kuffler and

Yoshikami, 1975) and the simultaneous recording of pre-and post-synaptic currents that has been achieved at certain unusually large synapses, including the squid giant synapse (Llinas et al., 1981), the rat calyx of Held (Borst and Sakmann, 1996) and frog embryonic neuromuscular junctions, *in vitro* (Yazejian et al., 1997). Nevertheless, because the dimensions of most nerve terminals preclude the direct recording of biophysical correlates of neurotransmitter release, information about synaptic vesicle exocytosis usually is gleaned indirectly by recording post-synaptic responses to released transmitter. An obvious caveat for such work is the need to ensure that experimental manipulations targeting the release process do not affect the post-synaptic sensitivity to released transmitter. Additionally, as has been observed at some synapses (Sun and Wu, 2001), saturating amounts of released transmitter can lead to underestimates of the number of synaptic vesicles that contribute to a secretory event. Nevertheless, with adequate controls, electrophysiological analyses will continue to provide vital insights into mechanisms of synaptic transmission.

In addition to the electrophysiological approaches noted above, transmitter release also has been studied by using other dynamic approaches, including electrochemical detection and capacitance measurements. Use of the former is restricted to synapses that release oxidizable neurotransmitters. A prominent example of this approach is the examination of serotonin discharge from leech motor neurons (Bruns and Jahn, 1995). This technology has also been applied to vertebrate central neurons and thorough reviews of data acquisition, analysis and outcomes are given by Mosharov and Sulzer (2005), Wightman (2006) and Robinson and colleagues (2008). Similarly, although capacitance measurements were initially restricted to studies of exocytosis in cells with large secretory granules, applications were later developed for cells with suitably large nerve terminals (Lindau et al., 1992; Sun and Wu, 2001; Sun et al., 2002; Klyachko and Jackson, 2002, Wolfel and Schneggenburger, 2003). A unique feature of capacitance measurements is that they provide insight into the properties of the exocytotic fusion pore and subsequent endocytotic events that are not accessible by using other techniques (Henkel and Almers, 1997; Lindau and Alvarez de Toledo, 2003; Jackson and Chapman, 2008). However, the small number of nerve terminals that can be subjected to capacitance measurements has restricted the utility of this methodology.

2.2.3 *Genetic Perturbation Strategies*:

Genetic manipulations continue to be among the most powerful approaches for identifying and characterizing the role(s) of proteins that participate in regulated exocytosis at nerve terminals (Augustine et al., 1996; Wu and Bellen, 1997; Littleton, et al., 1999; Fernandez-Chacon and Sudhof, 1999; Schwarz, 2006). In general, these experiments begin with a strategy for perturbing the expression or properties of the native protein followed by the use of biophysical or imaging techniques to assess the impact on synaptic function. For instance, an early screen for mutations affecting the movement of the nematode worm, *Caenorhabditis elegans*, led to the identification of a number of genes encoding proteins of the nerve terminal secretory apparatus (Brenner, 1974; Richmond, 2005; Barclay et al., 2012). An interesting extension of this technology is the use of RNAi screens that can be administered *in vivo* both in C. elegans and in Drosophila (Fraser et al., 2000; Dietzl et al., 2007; Barclay et al., 2012). In addition to such screens, a cornucopia of other genetic strategies is now in use. These include: knockouts (KOs), conditional KOs, transgenics (including, knockins; indeed, knockin of the wild-type gene is often used as a test for the specificity of the KO phenotype), knockdowns (typically, using antisense RNA or RNAi) and over-expression methods. It was noted above that one of the challenges of interpreting data from these experiments is the propensity for genetic manipulations to induce compensatory changes in the expression or properties of other proteins. Among the better examples of this phenomenon are the substitution of Cav2.2 (N-type) and Cav2.3 (R-type) Ca^{2+} channels for Cav2.1 (P/Q-type) Ca^{2+} channels at the neuromuscular junction of mice deficient in the α-subunit of Cav2.1 (P/Q-type) Ca^{2+} channels (Urbano et al., 2003) and the replacement of SNAP-25 with SNAP-24 in SNAP-25 null alleles of Drosophila (Vilinsky et al., 2002). However, in most circumstances, if compensatory changes do occur, they are not as clear cut as in these examples. Nevertheless, it has become commonplace for investigators to assess the relative expression level of a number of other presynaptic proteins in situations where compensatory changes might be expected, and these control experiments have helped to constrain alternative interpretations of the data. Likewise, in situations where genetic knockins are used, it is important to determine the subcellular distribution and expression level of the knockin gene product relative to the native protein. Although such controls are important for the interpretation of the data, often it is difficult to make the requisite measurements. For instance, as noted earlier, there are large differences in the published estimates of the number of synaptobrevin 2 and synaptotagmin I molecules on synaptic vesicles (Takamori et al., 2006; Mutch et al., 2012). Given these disparities, it

should be evident that genetic manipulation of synaptic vesicle proteins might produce substantial changes in the copy number of synaptic vesicle proteins that would be difficult to detect. Future technological advances in detecting and quantifying specific proteins at nerve endings should help to alleviate this concern. Finally, the interpretation of genetic perturbations is also frequently complicated by the multiple roles of proteins of the secretory apparatus. Later in this review we will allude to the multiple functions of synaptotagmin 1 (in Ca^{2+} sensing and endocytosis) and syntaxin 1a (in docking and fusion), and the challenge this poses for distinguishing the relative contributions of these functions to the phenotype observed after a genetic manipulation. Nevertheless, many of the preceding concerns have been diminished by using KO animals into which mutated versions of the native gene product are introduced. Later discussions will highlight data from several of these KO-knockin experiments.

2.2.4 *Other Perturbation Strategies:*

This category includes the use of antibodies, recombinant proteins, peptides, toxins, drugs and other agents to alter transmitter release parameters. For instance, early studies using antibodies against SNARE proteins and synaptotagmin provided some of the first corroborative evidence supporting the crucial role of these proteins in transmitter release at nerve endings (Mikoshiba et al., 1995; Mochida et al., 1995, 1997; Sugimori et al., 1998). Similarly, investigations targeting the squid giant synapse used recombinant fragments or peptide sequences from these same proteins to modulate transmitter release (Bommert et al., 1993; Hunt et al., 1994; O'Connor et al., 1997). A review (Augustine et al., 1996) provides a thorough discussion of the pros and cons of these approaches. Clearly, over the last decade, it has become more common to use viral expression of native or mutated forms of recombinant proteins in neurons, *in vitro* or *in vivo* (Finley et al., 2002; Stevens and Sullivan, 2003; Deak et al., 2006; Delgado-Martinez et al., 2007; Young and Neher, 2009; Kochubey and Schneggenburger, 2011). A general concern in these experiments is that the high level of protein expression often achieved may influence the results in ways that are difficult to control.

Protein toxins have also made major contributions to our understanding of exocytotic mechanisms at nerve terminals. In addition to the peptide toxins that block presynaptic Ca^{2+} channels, clostridial neurotoxins and α-latrotoxin (the primary toxic agent of black widow spider venom), have been valuable

probes of presynaptic function (Davletov et al., 2012). In particular, the clostridial neurotoxins (tetanus toxin and the seven isoforms of botulinum toxin) were discovered to be highly selective endoproteases that cleave specific members of the SNARE family (Schiavo et al., 1992; 1993,a,b; Link et al., 1992, Pellizzari et al., 1999). The strong correlation between the selective proteolytic cleavage of individual SNARE proteins and the inhibition of stimulus-evoked neurotransmitter release remains one of the most compelling arguments for the crucial role of SNAREs in exocytotic transmitter release (Schiavo et al., 2000; Sorensen, 2009; Sudhof and Rothman, 2009; Pantano and Montecucco, 2013). In contrast to the clostridial toxins, α-latrotoxin triggers massive transmitter release at a wide range of synapses, and although its receptors appear not to contribute directly to the exocytotic process itself, this toxin remains a prominent tool for studying the release process (Deak et al., 2009; Davletov et al., 2012).

A disparate array of small molecule drugs and other agents can profoundly alter transmitter release at nerve endings. For instance, elevated osmotic pressure augments quantal transmitter release at frog motor nerve terminals (Furshpan,1956), and subsequently has become a widely used tool to estimate the readily releasable pool of synaptic vesicles (Rosenmund and Stevens, 1996). In contrast, although La^{3+} inhibits stimulus-evoked transmitter release, it triggers a profound increase in the frequency of spontaneous quantal discharges at frog neuromuscular junctions (Heuser and Miledi, 1971) and elsewhere (Chung et al., 2008). A drug (LY294002) that inhibits various kinases also induces a ~100-fold increase in spontaneous quantal events at frog nerve terminals (Rizzoli and Betz, 2002). Alternatively, agents that activate members of the protein kinase C family typically enhance evoked transmitter release at synapses (Majewski and Iannazzo, 1998). Finally, inositol polyphosphates inhibit evoked transmitter release at the squid giant synapse (Fukuda et al., 1995), and in hippocampal neurons (Yang et al., 2012). For many of these agents, the specific molecular target(s) of their action remain(s) unclear. However, as exemplified by the inositol polyphosphates, which appear to interact with the C2B domain of synaptotagmin (Llinas et al., 1994; Fukuda et al., 1995; Yang et al., 2012), the more we learn about the secretory machinery, the better equipped we will be to understand these agents and their actions.

2.2.5 *Reconstitution of Exocytosis in Cell-Free Systems Using Proteoliposomes*:

One of the consummate arguments in support of SNARE proteins as triggers of exocytotic membrane fusion is that reconstitution of recombinant SNAREs in liposomes leads to enhanced membrane fusion, *in vitro*. This outcome was first reported by Weber and colleagues (1998), and numerous groups have since exploited this approach to enrich our insight into the types of protein interactions that regulate and mediate membrane fusion. The following discussion will focus first on technical variables influencing the conduct of these experiments and then segue into issues of data interpretation.

As noted earlier (in 2.1.2), assays were originally developed to monitor the fusion of artificial bilayer membranes composed exclusively of phospholipids, or other biologically important lipids, including cholesterol. Thus, the fundamental quest in studies using proteoliposomes is to determine whether the inclusion of protein enhances the basal rate of liposomal membrane fusion. In the original work with SNAREs (Weber et al., 1998), the fusion assay monitored the de-quenching of fluorescently labeled lipids. This de-quenching occurred when liposomes bearing the v-SNARE, synaptobrevin, and fluorescently labeled phospholipids were mixed with liposomes containing the t-SNAREs and no labeled lipids. Suitable interactions between v-SNARE liposomes and t-SNARE liposomes led to de-quenching of the reporter lipid. Subsequently, it has been emphasized that these so-called lipid-mixing assays are not ideal reporters of membrane fusion, because lipid mixing can occur between apposed liposomes without overt membrane fusion (Wang et al., 2009; Chan et al., 2009; Kyoung et al., 2011, 2013; Diao, et al., 2013). To provide a more-direct index of membrane fusion, procedures have been developed which use evidence of content mixing along with, or independently of, lipid mixing (Wang et al., 2009; Chan et al., 2009; Kyoung et al., 2013; Diao, et al., 2013). Thus, it is important to keep in mind that studies which rely exclusively on lipid mixing may not accurately portray the extent of membrane fusion. Clearly, this consideration complicates the interpretation of the results in numerous earlier publications.

In addition to concerns about the fusion assay employed, there are a number of other variables that can influence the outcome of proteoliposome fusion assays. As with experiments using protein-free liposomes, the lipid composition and dimensions of the vesicles can affect the results, as can the temperature of the experiment and the composition of the medium in which the vesicles are suspended (Dennison et al., 2006; Chen et al., 2006; Chan et al., 2009). More importantly, there often is variability among groups in the nature of the recombinant protein that is incorporated into liposomes. These

variables include differing protein purity, residual amino acid residues that remain attached after removal of the fusion protein, and the status of post-translational modifications. Additionally, there is variation in the lipid:protein ratio that is used when preparing proteoliposomes. This latter issue is further complicated by disagreements regarding the biologically relevant level of protein to use. For instance, should one target the 70 copies per synaptic vesicle figure for synaptobrevin 2 endorsed by Takamori and co-workers (2006) or the ~10/vesicle estimate of Mutch and co-workers (2011)? Nevertheless, in spite of these concerns, progress continues to be made in developing creative solutions for these issues, and as a tool for gauging the potential roles of secretory proteins, this technology will undoubtedly continue to make important contributions.

The results of the vast majority of proteoliposomal fusion assays have been interpreted in the context of Ca^{2+}-dependent, synaptic vesicle exocytosis at nerve terminals. Currently, these interpretations suffer from two fatal flaws. First, very few of these investigations have used full-length synaptotagmin I or II, which are the biologically relevant Ca^{2+} sensors for fast exocytosis at most nerve terminals. Second, membrane fusion in all of the currently published assays is at least one order of magnitude slower than what is observed at nerve endings. With respect to the first criticism, a promising effort was made recently to obtain recombinant synaptotagmin I in a form that is much closer to its *in vivo* state (Vrljic et al., 2011). However, it still was not established whether this recombinant synaptotagmin was fatty acylated in the same manner as the native protein. The potential importance of this issue will be addressed in section 2.3.2.4. The second criticism is related to the original discussion of the very short delay (60-200 μsec) between the Ca^{2+} signal and exocytosis at nerve terminals. To date, the fastest proteoliposomal fusion assays have reported events that occur in the msec time domain (Wang et al., 2009; Domanska et al., 2009). Although such kinetics may be adequate to account for regulated exocytosis in cells releasing hormones or other substances (Martin, 2003), they clearly are of negligible relevance to nerve endings. Indeed, a straightforward conclusion can be drawn from the kinetics of membrane fusion reported in the reconstitution studies published to date: specifically, it is likely that these experiments are deficient in one or more components that are necessary to achieve the fast kinetics of secretion at nerve endings. An explicit hypothesis concerning the identity of this missing component will be discussed later (section 2.7).

2.2.6 *Other Approaches to the Study of the Secretory Apparatus*:

A diverse array of other experimental techniques has been deployed to provide insight into secretory events at nerve terminals. Among these approaches are:

Chemical assays of transmitter release: From the time of Loewi's famous identification of acetylcholine as the parasympathetic neurotransmitter in the heart, direct (and, indirect) chemical assays of neurotransmitter release have been performed using an astonishingly diverse range of procedures. For instance, radioisotopic labeling of neurotransmitters or their precursors has been widely used in neurochemical studies. And, quantitative assays for acetylcholine have been devised which rely on mass spectrometry (Jenden et al., 1973; Polak and Molenaar, 1979). But, acetylcholine release has also been detected by using patch clamp pipettes with acetylcholine receptors trapped in a membrane patch (Young and Poo, 1983; Hume et al., 1983), or by collecting photons from the oxidation of choline following the action of acetylcholinesterase (Stanley, 1993). We also alluded previously to the development of techniques for the electrochemical detection of biogenic amines, *in vivo* (Wightman, 2006; Robinson et al., 2008). And, other groups have developed methods to detect neuronal glutamate release using electrode-based approaches (Yu et al., 2011; Tolosa et al., 2013). The importance of these investigations is that they remain useful for validating the chemical identity of the signaling agent at specific synapses. However, because of the greater sensitivity and temporal resolution of electrophysiological approaches, chemical techniques tend to be used less frequently for studies of synaptic function.

Biochemical characterization of exocytotic proteins: Procedures for purifying synaptic vesicles were developed within a decade of the discovery of these organelles (DeRobertis et al., 1963; Whittaker, et al., 1964). A serendipitous development that attended these efforts was the finding that homogenization of mammalian brain in isosmotic sucrose solution led to the formation of largely intact presynaptic structures referred to as synaptosomes (Gray and Whittaker, 1962). Synaptosomes have been widely used for studies of the synthesis, storage, release and re-uptake of neurotransmitters (Whittaker, 1965; Snyder, 1970). However, it took significantly longer for protein components of synaptic vesicles and synaptosomes to be identified. An important contribution to this effort was the acquisition of antibodies targeting specific proteins. For instance, synaptophysin (p38) antibody (Wiedenmann and Franke, 1985; Jahn et al., 1985) played an important role in the purification of this synaptic vesicle constituent (Rehm et al., 1986). Likewise, monoclonal antibodies obtained using purified synaptic vesicles as antigens led to

the identification of such novel vesicle components as p65 (later re-named, synaptotagmin) and SV2 (Matthew et al., 1981; Buckley and Kelly, 1985). In parallel, synapsin I was originally identified as a nerve terminal phosphoprotein, and was subsequently found to associate electrostatically with synaptic vesicles (Huttner et al., 1983; De Camilli et al., 1983).

These early biochemical studies were followed by developments in a number of related areas. For instance, in addition to phosphorylation, other post-translational modifications of these proteins were documented, such as the fatty acylation of synaptotagmin (Chapman et al., 1996; Veit et al., 1996) and synaptobrevin (Veit et al., 2000). With the advent of molecular cloning, cDNAs encoding many synaptic vesicle proteins were sequenced and related family members were identified (McCaffery and DeGennaro, 1986; Sudhof et al., 1987a,b, 1989; Buckley et al., 1987; Trimble et al., 1988; Elferink et al., 1989; Baumert et al., 1989; Perin et al., 1990, 1991b; Bajjalieh et al., 1992). And, several groups used synaptic vesicle proteins as “bait” to search for interacting macromolecules. Prominently, this approach led to the discovery of syntaxin (Bennett et al., 1992), a plasma membrane protein that interacted with synaptotagmin. Soon after its discovery, syntaxin was recognized as being a key component of the SNARE complex (Sollner et al., 1993a). Collectively, these approaches led to the identification of most of the proteins that are now regarded as playing important roles in the Ca^{2+}-dependent triggering of synaptic vesicle exocytosis.

A major addition to the biochemist’s repertoire in the last decade is proteomic inventories of proteins associated with synaptosomes (Bai and Witzmann, 2007; Filiou et al., 2010) and synaptic vesicles (Coughenour et al., 2004; Morciano et al., 2005; Takamori et al., 2006). Although there has been considerable convergence of results among the synaptic vesicle proteomics studies (Burre and Volknandt, 2007), much work remains to be done to establish the minimum complement of proteins required to produce a synaptic vesicle that is competent to participate in fast exocytosis. Toward this goal, it certainly would be helpful to undertake analyses of the relatively more-homogeneous synaptic vesicles from marine electric organs (Kelly et al., 1979). Nevertheless, because synaptic vesicles undergo cycles of exocytosis, endocytosis and recycling, there are also likely to be many transient protein-vesicle interactions whose role will be a challenge to clarify.

The proteomic studies to date provide information concerning an “average” synaptic vesicle. A complementary approach (Mutch et al., 2011) looks at the dispersion within the vesicle population in the number of copies per vesicle of specific proteins. Interestingly, some proteins (like, synaptotagmin) show very little inter-vesicle variation in copy number, while others (like, synaptobrevin 2) exhibit considerable variability (Mutch et al., 2011). Future application of this technology (ideally, in conjunction with high-resolution freeze-fracture or tomographic reconstruction analyses) should be useful for helping to understand the factors that influence the stoichiometry of synaptic vesicle proteins, as well as the functional consequences of variations in this stoichiometry. Again, applying this technology to vesicles from electric organs could be quite informative.

NMR, X-ray crystallographic and other structural analyses: Many of the proteins implicated in regulated exocytosis have had their structure determined at atomic or nearly atomic dimensions. These data have been particularly important for understanding the interactions that stabilize the four-helical SNARE bundle (Sutton et al., 1998; Fasshauer et al., 1998 a,b; Ernst and Brunger,2003; Poirer et al., 1998; Stein et al., 2009), as well as the binding of complexin to the SNARE complex (Chen et al., 2002). Furthermore, X-ray and NMR data were instrumental in identifying the residues involved in Ca^{2+} binding by the C2A domain of synaptotagmin I and for revealing that Ca^{2+} binding does not induce a significant change in the conformation of this region (Sutton, et al., 1995; Shao et al., 1998). This latter conclusion also holds for Ca^{2+} binding to the C2B domain of synaptotagmin I (Fernandez et al., 2001; Ubach et al., 2001). However, although synaptotagmin III structure was obtained at high resolution (Sutton et al., 1999), the X-ray structures of native, full-length synaptotagmin I and II have not been solved. For reasons that will be addressed later (section 2.7), such structural results are likely to have important implications for understanding the role of synaptotagmins I and II in exocytotic membrane fusion. Finally, spectroscopic and biophysical investigations have revealed an unusual tilt in the membrane-spanning domain of synaptobrevin (Bowen and Brunger, 2006), while Xu and colleagues (2013) have extended our understanding of the interactions among SNAREs, synaptotagmin and complexins. However, the relevance of these latter data to exocytotic triggering at nerve terminals remains to be established.

Secretory proteins in non-neuronal cells: Important contributions to our understanding of regulated exocytosis have come from studies of secretory events in non-neuronal cells (Martin, 2003). For instance,

the pheochromocytoma cell line, PC12, has been used by many groups to investigate the function of specific components of the exocytotic machinery (Shoji-Kasai et al., 1992; Elferink et al., 1993). However, because the kinetics of secretion are appreciably slower in non-neuronal secretory cells (Martin, 2003), this review focuses almost exclusively on data obtained in studies of fast, synchronous exocytosis in neurons. The rationale for this decision is that the evolutionary adaptations that presumably were made to achieve the exceptionally rapid coupling between Ca^{2+} entry and synaptic vesicle exocytosis may not be conserved in other secretory pathways. Of course, once a better understanding of rapid exocytosis at nerve terminals is achieved, it will be easier to identify specific molecular differences between events at nerve terminals and elsewhere.

2.3 Synaptotagmins/SNAREs and their roles in fast, Ca^{2+}-dependent exocytosis at nerve terminals

As a prelude to reviewing the data supporting the central roles of synaptotagmins I and II, synaptobrevin 2, syntaxin 1a and SNAP-25 in fast, synchronous transmitter release at nerve endings, it is important to point out that most investigations of the secretory role(s) of these proteins have monitored their contributions to at least two distinct exocytotic pathways at nerve terminals: the spontaneous (stimulus-independent) quantal release of transmitter which is detected at virtually all normal synapses (as originally reported by Fatt and Katz, 1952), and the action potential (and, Ca2+)-dependent, synchronous release of transmitter which is the major focus of this review. These two discrete forms of transmitter secretion will be referred to as “spontaneous” vs. “stimulus-dependent”, “synchronous”, or “evoked”. However, nerve impulses can also trigger a form of exocytotic transmitter release referred to as “asynchronous” secretion in which quantal discharges are detected for tens to hundreds of milliseconds after an action potential (Goda and Stevens, 1994). A good example of asynchronous transmitter release is seen in hippocampal neurons after KO of the synaptotagmin I gene in mice (Geppert et al., 1994), and zebrafish exhibit robust asynchronous release (Wen et al., 2010). However, the precise cellular and molecular relationships among spontaneous, synchronous and

asynchronous secretion remain unclear. Nevertheless, many investigators also use the application of hypertonic sucrose solutions to assess the “readily-releasable” pool of synaptic vesicles (Rosenmund and Stevens, 1996). This asynchronous form of quantal secretion is Ca^{2+}-independent, and, not surprisingly, is largely unaffected in synaptotagmin I KO mice (Geppert et al., 1994). However, the molecular mechanism by which sucrose exerts this effect is not yet understood. Some investigators also evaluate the contribution of exocytotic proteins to manifestations of short-term synaptic plasticity (such as, facilitation and depression), as well as the responsiveness to certain pharmacological treatments (including, Ca^{2+} ionophores or the spider venom toxin, α-latrotoxin). Finally, to test for the possible impact of experimental manipulations on synaptic vesicle recycling, electron microscopy and FM-dye experiments are often conducted. Taken together, these approaches constitute the primary arsenal for assessing the *in vivo* roles of the core exocytotic proteins.

At least two complicating features attend these efforts. The first is the fact that “exocytotic” proteins tend to have multiple roles (Jahn and Fasshauer, 2012). For instance, in addition to serving as Ca^{2+} sensors for regulated exocytosis at nerve terminals, some members of the synaptotagmin family also contribute to the endocytotic recycling of synaptic vesicles (Zhang et al., 1994; Jorgensen et al., 1995; Poskanzer et al., 2003). Thus, in principle, experiments in which synaptotagmin function is perturbed *in vivo* need to distinguish functional outcomes that derive from changes in Ca^{2+} sensing from those influencing vesicle recycling. Similarly, syntaxins were originally identified as synaptotagmin-interacting proteins (Bennett et al., 1992) and are now recognized as crucial components of the trimeric SNARE complex (Figure 1). However, syntaxins may also have a critical role in pre-fusion steps during which they interact with munc-18/unc-18 (Toonen and Verhage, 2007; Sudhof and Rothman, 2009; Sorensen, 2009; Barclay et al., 2012), and modulate the function of voltage-gated Ca^{2+} channels (Bezprozvanny et al.,1995; Wiser et al., 1996) . Again, this multiplicity of functions poses challenges for those seeking to discriminate which role of syntaxin predominates in a specific

experimental setting. Hence, it is critically important to keep in mind alternative explanations of experimental data when evaluating the function(s) of protein components of the exocytotic machinery.

The second complicating factor is: In general, integral membrane proteins, including members of the syntaxin, synaptobrevin and synaptotagmin families must be translated and processed in the neuronal cell body before export to nerve terminals. Clearly, this facet of protein biosynthesis means that in addition to the nerve terminal, most proteins of the secretory apparatus will also be detectable in the neuronal cell body and along or within the axon. Another important consideration is that some experimental strategies may affect the subcellular distribution of secretory proteins in phenotypically interesting ways. Thus, when investigators undertake mutagenesis studies or other perturbation strategies (including, knockins), it is important to determine whether the modified form of the protein achieves a comparable level of expression and distribution as the wild-type form. Although some investigations go to considerable lengths to control for protein expression and distribution within the neuron (for example, see work with synaptotagmin by Paddock et al., 2011), not all studies include such controls. Consequently, the interpretation of some data sets may be confounded by uncertainty regarding the level or distribution of the expressed protein.

With the preceding caveats, the following discussion begins by reviewing data supporting the role of synaptotagmin family members (particularly, synaptotagmins I and II) as Ca^{2+} sensors for the synchronous release of transmitter at nerve endings. This will be followed by a consideration of the postulated role of SNAREs as the catalysts of membrane fusion at nerve terminals. Because several different schemes have been invoked to explain the fusion-promoting role of SNAREs (Fig.3), we will also address empirical approaches that could help to distinguish among these alternatives. Similarly, the precise role by which synaptotagmin regulates SNARE-driven membrane fusion remains uncertain, and a separate sub-section will address current

ideas for how this regulation is achieved. A prominent theme throughout this discussion will be the shortcomings of current models of SNARE-mediated, synchronous neurotransmitter release at nerve endings. Indeed, it was these shortcomings that led to the development of an alternative model of this process. The “dyad” hypothesis proposes that a quartet of synaptotagmins at the vesicle-plasma membrane interface serve as Ca^{2+} sensors and membrane fusion catalysts (Gundersen and Umbach, 2013). Later sections compare and contrast the dyad scheme with SNARE-based models of membrane fusion.

2.3.1 Synaptotagmins I and II as the Ca^{2+} sensors for fast, synchronous exocytosis at nerve endings:

Seminal observations by Perin and colleagues started the synaptotagmin stampede. They cloned and sequenced the cDNA for rat p65 (Perin et al., 1990), a protein previously shown to be an intrinsic component of synaptic vesicles (Matthew et al., 1981). The deduced amino acid sequence of p65 included a single membrane-spanning domain and a pair of C2 domains, which were previously recognized as Ca^{2+}-binding modules in members of the protein kinase C (PKC) family. Moreover, like PKC family members, bacterially expressed fragments of p65 that contained the C2 domains bound radioactive phosphatidyl serine, but not phosphatidyl choline (Perin et al., 1990). Further evidence for the prospective role of synaptotagmin’s C2 domains came from the finding that they mediated the Ca^{2+}-dependent binding of synaptotagmin to liposomes prepared using negatively charged phospholipids (Brose et al., 1992). Because this binding occurred in a physiologically relevant range of Ca^{2+} (10-100 μM), these observations helped to elevate the profile of synaptotagmin above that of other Ca^{2+}-binding proteins of nerve terminals. For instance, based on the relatively modest effect of some calmodulin antagonists on transmitter secretion, calmodulin was discounted as being the primary Ca^{2+} sensor for nerve terminal exocytosis (Sahaf and Publicover, 1985; Schweitzer, 1987). Similarly, although the synaptic vesicle protein, synaptophysin, was found to be a Ca^{2+} binding protein (Rehm et al.,

1986), its cDNA sequence failed to reveal any known Ca^{2+}-binding domains (Buckley et al., 1987). Hence, synaptophysin was quickly overshadowed by synaptotagmin.

In parallel with the work discussed above, cloning by homology established that p65 was highly conserved from fruit flies to humans suggesting that it played a vital role at synapses (Perin et al., 1991b). In this same paper, Perin and colleagues (1991b) proposed that p65 be re-named synaptotagmin based on its subcellular location and binding properties. The first indications of a multi-gene family became apparent with the cloning and sequencing of the cDNA for rat synaptotagmin II (Geppert et al., 1991) and the presence of three distinct synaptotagmin cDNAs in a marine ray (Wendland et al., 1991). These cloning experiments propelled the next round of progress in understanding synaptotagmin's function.

Interestingly, early functional studies led to controversy regarding the biological role of synaptotagmin. For instance, PC12 cells deficient in synaptotagmin I remained capable of secreting catecholamines in response to stimuli (Shoji-Kasai et al., 1992). In contrast, Elferink and colleagues (1993) reported that injection of PC12 cells with synaptotagmin antibody impaired regulated exocytosis. Similarly, C2 domain peptides occluded transmitter release in squid without affecting the number of morphologically docked synaptic vesicles (Bommert et al., 1993). Meanwhile, early genetic studies using nematode worms and fruit flies (Nonet et al., 1993; DiAntonio et al., 1993) pointed to a role of synaptotagmin in synaptic function. However, because of persistent movement in null mutant embryos of both species, and ongoing spontaneous transmitter release at Drosophila larval neuromuscular junctions, it was concluded that synaptotagmin was not necessarily essential for regulated exocytosis. Later efforts revealed that some of the apparent discrepancies among these reports were likely to be attributable to the presence of multiple synaptotagmin gene products both in PC12 cells and in Drosophila.

Subsequent mutagenesis studies in Drosophila (Littleton et al., 1993; DiAntonio and Schwarz, 1994; Broadie et al., 1994) offered increasing support for synaptotagmin as the primary

exocytotic Ca^{2+} sensor at nerve endings. An initial analysis of partial loss-of-function and null alleles documented that the Ca^{2+}-dependence of evoked transmitter release at larval neuromuscular junctions was significantly reduced relative to wild-type (WT) controls. This work was extended by an assessment of the phenotype of a larger panel of synaptotagmin mutants. These data revealed changes in the Ca^{2+}-dependence and Ca^{2+} cooperativity of stimulus-dependent transmitter release that were consistent with the idea that synaptotagmin is the exocytotic Ca^{2+} sensor for synchronous transmitter release (Littleton et al., 1994; 2001; Yoshihara and Littleton, 2002).

In agreement with the fruit fly results, Ca^{2+}-dependent transmitter release was severely curtailed in cultures of hippocampal neurons obtained from synaptotagmin I knockout (KO) mice (Geppert et al., 1994). However, spontaneous transmitter release was unaffected in these same cells (Geppert et al., 1994). Two additional secretory processes were studied in the KO neurons. First, it was observed that the response to hyperosmotic sucrose was unaffected in neurons from the synaptotagmin KO mice. Second, the number of quanta discharged after application of α-latrotoxin was indistinguishable from WT cells. These data argued that both the readily releasable pool and the total available pool of synaptic vesicles were unaffected in the KO neurons. These results were important, because at about the time this KO paper appeared, synaptotagmin was found to be a binding partner for the clathrin-AP2 complex, which is involved in clathrin-mediated endocytosis (Zhang et al., 1994). The fact that synaptic vesicle pools were not detectably attenuated in the KO mice implied that the observed phenotype was not due to an impairment of vesicle recycling. Taken together, these findings significantly constrained the possible secretory functions of synaptotagmin I and pointed toward a role in exocytotic Ca^{2+} sensing. However, these experiments did not unequivocally link the Ca^{2+}-sensing function of synaptotagmin to exocytotic triggering, and several years passed before such results were obtained.

The strategy that ultimately led to widespread acceptance of synaptotagmin I as the primary Ca^{2+} sensor for regulated exocytosis at nerve endings relied on knockin technology in mice and fruit flies. The initial mouse experiments exploited a point mutation (R233Q) that diminished the Ca^{2+} affinity of the C2A domain by ~50% along with a control mutation (K236Q) that had no significant effect on Ca^{2+} binding (Fernandez-Chacon et al., 2001). Synaptic function was quantified by using hippocampal neurons from knockin animals that were cultured to form autapses. Among the salient observations were that vesicular release in the R233Q neurons was attenuated ~45% relative to K236Q or WT controls. However, the readily releasable pool of synaptic vesicles (as judged by using hypertonic sucrose) was unaltered in the R233Q mutant. More importantly, the relationship between the extracellular Ca^{2+} concentration and the amplitude of the stimulus-evoked responses in the R233Q mutant neurons was shifted to the right, as one would predict for a lower-affinity Ca^{2+} sensor. The R233Q mutants also took longer to respond to the release-promoting effects of a Ca^{2+} ionophore, a result consistent with the idea that it takes longer for cytosolic Ca^{2+} to reach the threshold necessary to trigger exocytosis. Collectively, these findings eliminated most of the resistance to the idea that synaptotagmin I is the principle Ca^{2+} sensor for nerve terminal exocytosis at a broad range of synapses.

Additional insight into synaptotagmin function *in vivo* has come from a series of knockin studies using Drosophila. Before discussing these results, it is important to note that an inadvertent mutation in the C2B domain of the original rat synaptotagmin I cDNA (glycine to aspartate) dramatically affected the folding of this module, and led to conflicting outcomes in studies that had previously assessed the role of this region (Ubach et al., 2001). By using the corrected sequence for the C2B domain, Ubach and associates (2001) found that, like the C2A domain, the C2B region also bound Ca^{2+}. The biological importance of Ca^{2+} binding to C2B was revealed in Drosophila knockin experiments (Mackler and Reist, 2002). Mackler and colleagues (2002) observed >95% reduction of evoked transmitter release when pairs of aspartate (D) residues were replaced with asparagines (N) in the Ca^{2+}-binding pockets of the C2B domain. These D to

N mutants also showed an appreciably reduced Ca^{2+}-sensitivity of stimulus-evoked transmitter release. Interestingly, because these mutations only targeted the C2B domain, the C2A domain was unaltered. Hence, these findings suggested that an intact C2A domain was insufficient for normal synaptotagmin function. This inference was consistent with reports (Robinson et al., 2002; Yoshihara et al., 2010) that mutations impairing Ca^{2+} binding to the C2A domain of Drosophila synaptotagmin do not significantly impair synchronous neurotransmitter release or the Ca^{2+}-dependence of transmitter release. Interestingly, work by Stevens and Sullivan (2003) and more recent results (Striegel et al., 2012) have tempered this conclusion (see below).

Further structure-function studies in Drosophila strengthened the evidence that Ca^{2+}-dependent interactions of synaptotagmin were crucial for stimulus-dependent transmitter release. For instance, Paddock and colleagues (2008) documented that a conserved basic residue in the C2A and C2B domains of synaptotagmin is important both for the Ca^{2+}-dependent membrane association of these regions and for normal evoked transmitter release. A second study by Paddock and colleagues (2011) provided evidence that synaptotagmin's C2 domains are designed to penetrate into a hydrophobic environment once they bind Ca^{2+}. Thus, when glutamate replaced an isoleucine residue in the C2B domain, it resulted in embryonic lethality even when co-expressed with native synaptotagmin. Moreover, evoked transmitter release at embryonic neuromuscular junctions of the C2B mutants was attenuated even more than in synaptotagmin null mutants. In contrast, replacing an equivalent hydrophobic residue in the C2A domain led to organisms exhibiting only about a 50% reduction of evoked transmitter release. These results were interpreted as pointing toward a critical role for this hydrophobic residue in enabling the membrane insertion of the C2B domain during exocytotic triggering with a permissive role for the C2A domain (Paddock et al., 2011).

Independent mutational analyses in Drosophila and mouse have further confirmed the crucial contribution of the C2A and C2B domains to the synchronous Ca^{2+}-dependent triggering of

exocytosis (Nishiki and Augustine, 2004; Rhee et al., 2005; Yoshihara et al., 2010). Prominently, Rhee and colleagues (2005) reported that tryptophan mutations of residues in the C2A and C2B domains of synaptotagmin I enhanced the Ca^{2+} affinity of synaptotagmin I and produced a corresponding increase in the Ca^{2+} sensitivity of evoked transmitter release at synapses formed by cultured mouse neurons. However, perhaps the most interesting recent development is that a reconsideration of the Ca^{2+}-binding role of the C2A domain revealed that previous mutations in which aspartate residues had been replaced by asparagines apparently had the unintended consequence of mimicking Ca^{2+} binding (Striegel, et al., 2012). Instead, if one replaces aspartate with glutamate, which impairs Ca^{2+} binding without charge neutralization, one observes a large (~80%) decline in synchronous transmitter release (Striegel et al., 2012). Although these data indicate that both C2 domains of synaptotagmin are necessary for the normal, synchronous release of neurotransmitter, they still support the conclusion that the C2B domain is the more important of the two modules. And, the overarching conclusion from all of these investigations is that synaptotagmin I is the primary Ca^{2+} sensor for fast, synchronous transmitter at a broad range of synapses. For further discussion see the reviews by Sudhof (2012), and Jahn and Fasshauer (2012).

The key question left unanswered by the preceding commentary is, “Once Ca^{2+} binds to the C2 domains of synaptotagmin, what happens next?” The majority of current models invoke a role for synaptotagmin in regulating SNARE-dependent membrane fusion (Sudhof, 2012; Jahn and Fasshauer, 2012). In addition, another protein, complexin, may play an important role in this process by virtue of its interaction with the assembled SNARE complex (McMahon et al., 1995; reviewed in Sudhof, 2012; Jahn and Fasshauer, 2012). However, there is little question that synaptotagmin transduces the Ca^{2+} signal that leads to synaptic vesicle exocytosis. To set the stage for addressing the proposed regulatory role of synaptotagmin, it is first necessary to scrutinize the SNAREs, SNARE complexes, the SNARE hypothesis and the four general models of SNARE-mediated membrane fusion (Fig.3).

2.3.2 SNARES, the SNARE Hypothesis and Models of SNARE-mediated Membrane Fusion:

The early 1990s witnessed several watershed moments in our understanding of the molecular mechanisms of regulated exocytosis at nerve terminals. In addition to the identification of synaptotagmin as a Ca^{2+} and phospholipid-binding protein of synaptic vesicles (Perin et al., 1990; Brose et al., 1992), tetanus and type B botulinum toxins were shown to be highly selective endoproteases that cleaved the synaptic vesicle protein, synaptobrevin2/VAMP2 (Schiavo et al., 1992; Link et al., 1992). Similarly, types A and D botulinum toxins were found to cleave SNAP-25 (Schiavo et al., 1993a). Because these clostridial neurotoxins were well known for their potent inhibition of neuromuscular transmission, the fact that they cleaved proteins associated with synaptic vesicles or the synaptic plasma membrane certainly suggested that these events were linked. Within this same time frame, the plasma membrane protein, syntaxin was identified as a binding partner for synaptotagmin (Bennett et al., 1992) which led to speculation that this interaction might be important for vesicle docking or fusion.

The investigation that tied together many of these disparate threads began with efforts to identify proteins required for the specificity of intracisternal Golgi transport. In addition to a soluble protein designated NSF (for N-ethylmaleimide sensitive factor), a trio of accessory proteins was isolated (referred to as soluble, NSF attachment proteins, or SNAPs) that enabled the interaction of NSF with Golgi membranes. The next step in this biochemical tour de force involved efforts to purify the membrane receptors for the SNAPs. Because SNAPs had been isolated from brain, efforts to identify the so-called SNAP receptors, or SNAREs, also used brain extracts (Sollner et al., 1993a). The remarkable outcome of these efforts was that the SNAREs comprised a trio of proteins previously known to reside at nerve terminals: synaptobrevin 2, syntaxins A/B and SNAP-25 (Sollner et al., 1993a). The convergence of these biochemical data with the neurotoxin results and the information about the subcellular distribution of these

proteins led to the conclusion that the SNARE proteins were likely to be major players in intracellular membrane trafficking in neurons and elsewhere (Sollner et al., 1993a).

In quick succession, it was demonstrated that synaptobrevin, syntaxin and SNAP-25 associated *in vitro* in a tight, heterotrimeric complex, the SNARE complex (Sollner et al., 1993b). Moreover, either α-SNAP or synaptotagmin could bind to this trimeric complex, and in the former case, when NSF was added, the SNARE complex could be dissociated in an ATP-dependent reaction (Sollner et al., 1993b). These observations gave impetus to the idea that the SNAREs and NSF might participate directly in the membrane fusion process (Sollner et al., 1993b). The model that was advanced assumed that the v-SNARE, synaptobrevin, interacted in anti-parallel fashion with the t-SNAREs, syntaxin and SNAP-25. Binding of synaptotagmin to this complex was postulated to serve as a fusion clamp (Sollner et al., 1993b; the notion of a molecular fusion clamp had just been propounded by Popov and Poo, 1993). Once synaptotagmin dissociated from this complex, α-SNAP could replace synaptotagmin to initiate dissociation of the SNAREs. The α-SNAP-NSF-dependent dissociation of the SNAREs was viewed as critical for fusion to proceed, because the presumed antiparallel interaction of the SNAREs clearly could not survive the topological re-arrangement that occurs during membrane fusion (Sollner et al., 1993b). Although this initial model has undergone substantial revision (Fig.3), it set the stage for further refinement of our understanding of biological membrane fusion and the role of SNAREs.

Chronologically, the next two major turning points came in 1997 and 1998. Structural studies revealed that SNARE complexes formed due to the coiling of single α-helical regions contributed by synaptobrevin and syntaxin along with a pair of α-helices from SNAP-25 (Hanson et al., 1997; Fasshauer et al., 1997; Sutton et al., 1998, Poirier et al., 1998). More importantly, the coiling of these helices occurred in a parallel orientation beginning at the N-end of each helix and proceeding to the C-end of each helix. The parallel arrangement of the components of the SNARE complex eliminated the topological problem inherent in the original, anti-parallel SNARE

model of Sollner and colleagues (1993b). Instead, it was recognized that the parallel interaction of the v-SNARE (synaptobrevin) with the t-SNAREs (syntaxin and SNAP-25) provided a mechanism to bring the synaptic vesicle membrane into very close proximity, or direct contact with the plasma membrane. Indeed, all subsequent SNARE-based models of membrane fusion are variations on this theme (Fig.3). Concurrently, the empirical evidence which ensured that SNAREs remained at the center of attention was published by Weber and colleagues in 1998. They demonstrated that the incorporation of recombinant, synaptobrevin 2 into one set of liposomes and t-SNAREs into a second set of liposomes enhanced the rate of lipid mixing between these liposomes (Weber et al., 1998). These findings had the dual effect of promoting liposomal fusion assays as a major vehicle for studying the role of proteins in membrane fusion, and they strengthened the evidence supporting the central role of SNAREs in this process.

At this point, the discussion will digress to consider independent lines of evidence that increasingly led to the acceptance of SNAREs as crucial contributors to biological membrane fusion. First, the previously noted correlation between the proteolytic cleavage of SNAREs by clostridial neurotoxins and the ensuing interruption of stimulus-evoked neurotransmitter release will be addressed in more detail. Second, mutagenesis studies made vital contributions to our understanding of SNARE function, *in vivo*. Third, acute perturbation experiments, notably using the squid giant synapse, lent early support to the SNARE hypothesis. And fourth, a huge effort was and continues to be invested in studying SNARE-mediated membrane fusion, *in vitro*. Below, considerable attention will be paid to the contributions of these strategies to the understanding of the mechanism of fast exocytosis at nerve terminals.

2.3.2.1 Cleavage of SNAREs by clostridial neurotoxins:

As noted previously, clostridial neurotoxins are endoproteases, and they specifically cleave the polypeptide backbone of SNARE proteins. For the most part, these cleavage sites occur within the α-helical “SNARE domain” of the target protein and inhibit the formation of the SNARE

complex (Pantano and Montecucco, 2013). The fact that there is a strong correlation between the toxin-dependent blockage of evoked transmitter release at synapses and the toxin-dependent impairment of SNARE complex formation *in vitro* is clear testimony to the importance of SNARE complexes for normal neurotransmission (Niemann et al., 1994; Montecucco and Schiavo, 1995; Humeau et al., 2000; Schiavo, et al., 2000). Indeed, this conclusion extends to all three SNARE proteins. Syntaxin is clipped once within its α-helical SNARE domain by botulinum toxin type C in a reaction that separates its membrane spanning domain from the rest of the protein (Schiavo et al., 1995). Similarly, synaptobrevin is the target for several of the clostridial neurotoxins which leave its membrane-spanning domain bereft of most of the crucial, helix-forming region (Pantano and Montecucco, 2013). Further, type E botulinum toxin removes ~20 residues from the C-terminus of SNAP-25 in a reaction that also impairs SNARE complex formation (Pantano and Montecucco, 2013). The lone exception concerns type A botulinum toxin and its action on SNAP-25. In most species, the type A toxin removes just 9 residues from the C-end of SNAP-25, yet this is enough to cause a long-lasting blockade of neuromuscular transmission (Pantano and Montecucco, 2013). As noted earlier, although SNARE complexes are generally thought to coil from the N-end of each helical region toward the C-end, there is evidence that the initial association between synaptobrevin and SNAP-25, which precedes SNARE complex formation, involves the C-end of SNAP-25 (Poirier et al., 1998). Thus, one possibility is that the type A toxin hinders SNARE complex assembly. Alternatively, the SNARE complex may still form, but may be destabilized (Sorensen et al., 2006). On the other hand, because the C-end of SNAP-25 participates in Ca^{2+}-dependent interactions with synaptotagmin in neurosecretory cells (Gerona et al., 2000), the loss of this interaction may contribute to the impairment of exocytosis. Nevertheless, further work will be needed to establish with certainty the mechanism by which this 9 residue truncation of SNAP-25 inhibits synaptic vesicle exocytosis (see Pantano and Montecucco, 2013 for an alternative explanation of this phenomenon).

Interpretation of these neurotoxin data is constrained by two caveats. First, in spite of the strong correlation between SNARE protein cleavage *in vitro* and the toxin-mediated impairment of neurotransmitter release, several groups have reported that intact SNAREs persist in preparations in which clostridial neurotoxin inhibition of transmitter release is complete (reviewed in Schiavo, et al., 2000). The prevailing explanation for this result is that there are pools of SNAREs that do not contribute to regulated exocytosis at nerve terminals and instead fulfill some other function (Schiavo et al., 2000). It will be important independently to verify this explanation. Second, the fact that transmitter release is impaired in toxin-treated preparations does not mean that SNAREs directly mediate the exocytotic event, per se. If SNAREs normally contribute to a critical step in the assembly or docking of "release ready" vesicles, toxin-mediated cleavage of SNAREs would have the same effect on the secretory process. Thus, additional evidence will be needed to define the precise step at which clostridial neurotoxins interfere with the transmitter release process.

2.3.2.2 Genetic studies of SNARE function in regulated exocytosis at nerve terminals:

Just as studies using synaptotagmin KO and knockin organisms have provided important insights into synaptotagmin function, genetic approaches have advanced our understanding of SNARE function in nerve terminal exocytosis. However, as the following summary will indicate, not all of the data are consistent with conventional views of the role of SNAREs in directly mediating membrane fusion, and abundant opportunities exist for further investigations.

An important consideration in reviewing these data is that although genetic strategies can be very powerful in identifying a pathway that is dependent on a particular protein, it often is difficult to make unambiguous molecular interpretations based on the phenotype of specific allelic variants. While this problem is exacerbated in situations in which compensatory changes in gene expression mask the mutant phenotype, the field of synaptic vesicle exocytosis has also witnessed some unexpected outcomes of genetic perturbation studies. For instance, one might

predict that if SNAREs were essential components of the machinery for mediating synchronous transmitter release, disruption of SNARE genes in mice should either be embryonically lethal or lead to rapid post-partum death (because movements necessary for breathing would not be possible). Although this prediction tends to hold true, a small cadre of syntaxin KO mice has been reported to survive to adulthood (McRory et al., 2008). Other unexpected phenomena include the observations that a small level of stimulus-dependent transmitter release persisted in mice with homozygous KO of the gene encoding synaptobrevin 2 (Schoch et al., 2001), while the frequency of spontaneous transmitter release events was elevated in SNAP-25 KO mice (Washbourne et al., 2002). These results contrast with the complete elimination of both spontaneous and stimulus-dependent transmitter release in cultured neurons from KO mice lacking either munc-13 or munc-18 genes (Verhage et al., 2000; Varoqueaux et al., 2002). These latter observations have created a bit of a conundrum, because they clearly represent the most severe “loss of quantal transmitter secretion” phenotype observed to date. However, munc-13 and munc-18 are widely assumed to play “pre-fusion” or regulatory roles in synaptic vesicle exocytosis (Toonen and Verhage, 2007; Sorensen, 2009; Barclay et al., 2012; Jahn and Fasshauer, 2012), so it will be interesting eventually to see how the molecular role of these proteins is reconciled with the genetic loss-of-function data.

2.3.2.2.1 Genetic analyses targeting synaptobrevin:

The initial studies of synaptobrevin hypomorphs and KO alleles in nematode worms were consistent with a role for synaptobrevin in neurotransmission (Nonet et al., 1998). More definitive insights into synaptobrevin function came from studies of Drosophila deficient in neuronal synaptobrevin. Deitcher and co-workers (1998) reported that although null mutants were embryonically lethal, useful data could be obtained from recordings at the embryonic neuromuscular junction. These experiments revealed a complete loss of stimulus-evoked transmitter release. However, spontaneous secretory events persisted at ~25% of the control

frequency. Interestingly, several other procedures (including, tetanic stimulation and challenge with depolarizing solutions, Ca^{2+} ionophore or black widow spider toxin) caused an increase of quantal discharges at the neuromuscular junction of the null mutants (Yoshihara et al., 1999). These data revealed a preferential blockade of synchronous transmitter release evoked by action potentials, and led to the proposal that synaptobrevin plays an important role in coupling Ca^{2+} entry to the fusion of synaptic vesicles.

The phenotype of synaptobrevin 2 KO mice was subsequently examined by Schoch and colleagues (2001). Homozygous KO animals died soon after birth. Electrophysiological analysis of synaptic events in cultured neurons from the KO animals revealed a profound decline (~100-fold) in stimulus-evoked transmitter release, as well as large (~10-fold) reductions in spontaneous and hypertonicity-induced secretion. The residual transmitter release seen in the KO neurons was presumed not to be due to the presence of other synaptobrevin isoforms (either synaptobrevin 1 or cellubrevin), which were anatomically undetectable in these cells. Interestingly, these results were interpreted by Schoch and colleagues (2001) as pointing to the possibility that, "…full SNARE complexes are not required for fusion as such but catalyze formation of transition states." Regardless, these results indicate that exocytotic events persist in synaptobrevin-deficient mice.

These initial observations were extended by studies examining the consequences of expressing cellubrevin or mutated forms of synaptobrevin 2 in the synaptobrevin 2 KO background (Deak et al., 2006). Interestingly, lentiviral expression of cellubrevin rescued stimulus-dependent transmitter release in the synaptobrevin 2 KO neurons. This observation raised the possibility that cellubrevin contributed to the residual evoked responses in the original synaptobrevin 2 KO neurons. To test this possibility, dual KO mice were selected and it was observed that small, stimulus-dependent responses persisted in neurons from mice deficient in both cellubrevin and synaptobrevin 2. Thus, cellubrevin was not responsible for the persistent evoked responses in

the synaptobrevin 2 KO neurons. However, insertions of 12 or 24 amino acid residues between the SNARE motif and the transmembrane domain of synaptobrevin 2 did produce unique outcomes. The 12 residue insertion led to a recovery of spontaneous transmitter release, but the readily releasable pools and recycling pools of synaptic vesicles were still greatly attenuated relative to WT controls. In addition, stimulus-evoked transmitter release was improved relative to KO neurons, but only to about 30% of the amplitude in WT neurons. Extending the linker to 24 residues led to no recovery of spontaneous quantal events, and a slight improvement of evoked responses to about 20% of WT neurons (versus ~8% in KO neurons). Collectively, these results indicate that the SNARE motif of synaptobrevin 2 needs to remain in close proximity to the membrane-spanning domain for the effective progression of synaptic vesicles through the exocytotic-endocytotic cycle. A final, important observation in this paper concerned a point mutation in synaptobrevin 2. Analyses based on the crystal structure of the SNARE complex had shown a glutamine (Q) residue in syntaxin 1a and SNAP-25 that associated with an arginine (R) of synaptobrevin 2 in the ionic (0) layer (Fasshauer et al., 1998). As a test of the prospective importance of this R residue of synaptobrevin 2, it was converted to a Q residue. Because the Q-synaptobrevin 2 rescued all of the measured parameters of synaptic function, at least in this empirical context, its functional importance was negligible.

A separate follow-up study of synaptobrevin 2 KO neurons *in vitro* provided further insight into the reduction of hypertonicity-induced secretion (Deak et al., 2004). Although synaptic vesicle number was not significantly reduced, the rate of replenishment of the readily releasable vesicle pool was slower as were the kinetics of uptake of such endocytotic markers as horseradish peroxidase and FM1-43. These results strongly suggested that synaptobrevin 2 contributes to the rapid recycling process that maintains the readily releasable pool of synaptic vesicles. The precise molecular role of synaptobrevin 2 in this process has not been delineated.

Further insight into the function of synaptobrevin 2 in vertebrate nerve terminals emerged from an analysis of the dual effects of lanthanides on quantal transmitter release in synaptobrevin 2- or synaptotagmin I-deficient neurons (Chung et al., 2008). This group found that millimolar concentrations of lanthanides triggered a rapid, Ca^{2+}-independent increase of spontaneous quantal events in synaptotagmin-deficient neurons, but not in synaptobrevin-deficient neurons. The opposite outcome was seen for the slow, Ca^{2+}-dependent increase of quantal secretion evoked by micromolar levels of lanthanide. Nominally, these results are consistent with the data from Drosophila showing that procedures which elevate cytosolic Ca^{2+} independently of activating presynaptic Ca^{2+} channels can trigger exocytosis in synaptobrevin-deficient neurons.

Another study that suggested a link between synaptobrevin and the normal Ca^{2+} sensitivity of synaptic vesicle exocytosis assessed the secretory phenotype at neuromuscular junctions of mice deficient in synaptobrevin 1 (Liu et al., 2011b). Although synaptobrevin 2 was still present at the mutant motor nerve terminals, stimulus-evoked transmitter release was blunted relative to WT controls. In addition, the Ca^{2+} cooperativity of evoked transmitter release was depressed. Clearly, additional work will be needed to clarify the molecular basis of these results which suggest that synaptobrevin influences exocytotic Ca^{2+} sensing.

2.3.2.2.2 Genetic analyses targeting SNAP-25:

The initial study of SNAP-25 mutant Drosophila examined a temperature-sensitive mutant allele (Rao et al., 2001). At neuromuscular junctions of third instar larvae, stimulus-evoked transmitter release was ~2-fold greater than WT controls and spontaneous transmitter release was ~6X the control level at the permissive temperature. However, at 37^{o} C, evoked transmitter release was depressed ~60% which is consistent with a role for SNAP-25 in transmitter secretion. A later study of this mutant (Kawasaki and Ordway, 2009) showed a significant depression of evoked responses at 33^{o} C, which was interpreted as being indicative of a role for SNAP-25 in exocytotic priming.

Further insight into SNAP-25 function came with the selection of Drosophila SNAP-25 null alleles (Vilinsky et al., 2002). These mutants die at the pharate adult stage at which point electroretinograms show a failure of synaptic transmission. Unexpectedly, neuromuscular transmission was found to be essentially normal in SNAP-25 null, third instar larvae (Vilinsky, et al., 2002). The explanation for this result is that SNAP-24 was found to have substituted functionally for SNAP-25 in these larvae. This outcome is among the better examples of the phenotypic impact of genetic compensatory mechanisms and points to the need for caution in interpreting the results of genetic manipulations where compensatory changes may affect the observed phenotype.

Deletion of the SNAP-25 gene in mice is embryonically lethal (Washbourne et al., 2002), and contrary to the situation with SNAP-24 in Drosophila, there was no sign of a compensatory up-regulation of the closely related, mouse SNAP-23 gene (note that deletion of the mouse SNAP-23 gene is early embryonically lethal; Suh et al., 2011). Functionally, stimulus-dependent transmitter release was abolished at neuromuscular junctions in the SNAP-25 KO mice, while the frequency and amplitude of spontaneous transmitter release events were increased. In central neurons, α-latrotoxin still triggered an increased frequency of quantal discharges, but depolarizing solutions did not promote exocytosis. These data are somewhat reminiscent of the mouse synaptobrevin KO data insofar as exocytotic events that are independent of depolarization-dependent Ca^{2+} entry are partially or largely spared, whereas the normal pathway for stimulus-evoked neurotransmitter release is profoundly impaired. Regardless, the embryonic lethality and the failure of neuromuscular transmission in the SNAP-25 KO mice point to the essential role of this protein.

Several studies have extended the original characterization of the mouse and Drosophila SNAP-25 KOs. Because the vertebrate SNAP-25 gene is alternatively spliced (a and b forms), Delgado-Martinez and colleagues (2007) asked whether different functional outcomes attended

the virally transduced expression of SNAP-23, SNAP-25a or SNAP-25b in central neurons from SNAP-25 KO mice. Among the interesting observations in this study were that hypertonic sucrose still triggered quantal discharges from SNAP-25 null neurons (~10% of control, which again is reminiscent of neurons from synaptobrevin 2 KO mice), and that SNAP-23 nearly restored this component of secretion to the level observed with SNAP-25a. Moreover, while synchronous transmitter release required SNAP-25a or SNAP-25b, the expression of SNAP-23 led to significant asynchronous release. Thus, at least where action potential-triggered transmitter release was concerned, SNAP-25 was essential and could not be replaced by SNAP-23.

Independent evidence for SNAP-25 function at nerve endings emerged from the discovery of a missense mutation in the SNAP-25 gene in the blind drunk mouse (Jeans et al., 2007). In these animals, a threonine for isoleucine substitution at residue 67 of SNAP-25b led to a reduction (~20%) in the amplitude of excitatory responses recorded in cortical slices. This decline was shown possibly to be linked to a decreased rate of replenishment of the readily releasable pool of synaptic vesicles. Although these data clearly implicate SNAP-25 in presynaptic function, it is challenging to draw mechanistic conclusions in a situation in which other compensatory mechanisms may be operating.

A direct comparison was made of the impact on synaptic function in neuronal cultures from SNAP-25 KO and synaptobrevin 2 KO mice (Bronk et al., 2007). Whereas field stimulation of cultured neurons evoked small synaptic responses in virtually all synaptobrevin 2 KO neurons, only about a third of SNAP-25 KO neurons showed a detectable response. Also, among the SNAP-25 KO neurons that responded to field stimulation, depression of synaptic responses was seen with repeated stimuli, whereas facilitation was present in synaptobrevin 2 KO neurons. Nevertheless, neurons from both KO mice showed similar reductions in spontaneous transmitter release and in the level of release triggered by hyperosmotic sucrose (~10% of WT neurons).

Although no comparison was made to synaptobrevin 2 KO cells, SNAP-25 KO neurons showed increased quantal secretion in the presence of a Ca^{2+} ionophore. However, the rate of quantal events was still a small fraction of the WT control level. Finally, SNAP-25 deficiency had no demonstrable effect on synaptic vesicle recycling beyond what would be expected for the low rates of exocytosis. Collectively, these data identified subtle *in vivo* differences between these two SNAREs in their contribution to secretion-related events at nerve terminals. Nevertheless, given the critical role proposed for SNAREs in regulated exocytosis (Fig.3), it is interesting that stimulus-dependent transmitter release events persist in these KO neurons.

Although a controversy had emerged concerning the role of SNAP-25 in transmitter release at excitatory versus inhibitory synapses, a paper by Tafoya and colleagues (2006) largely reconciled earlier differences by showing that SNAP-25 does function at both GABAergic and glutamatergic nerve terminals. Then, returning to molecular analyses of SNAP-25 function, Weber and colleagues (2010) examined the effects on spontaneous and evoked transmitter release of mutations targeting different regions of SNAP-25. Using viral transfection of hippocampal autaptic cultures from SNAP-25 KO mice, they observed that deleting 9 residues at the C-end of SNAP-25 (comparable to cleavage by type A botulinum toxin) abolished spontaneous release and reduced evoked responses by ~90%. Likewise, alanine substitutions in layers 7 and 8 near the C-end of the SNARE motifs of SNAP-25 blunted the recovery of evoked responses and spontaneous transmitter release. However, mutations that diminished interactions in the middle of the SNARE complex or at the N-end had very different effects from the C-end mutations. Removal of 24 N-end residues led to a 4-5-fold increase of spontaneous transmitter release, while evoked responses were indistinguishable from control. Interestingly, there was an impairment of the recovery of evoked responses after high frequency stimulation in cells with the N-end deletion. Mutations aimed at destabilizing the central region of the SNAP-25 SNARE motifs led to the recovery of normal evoked responses, while spontaneous secretion was elevated. Taken together, these data confirmed the important role of SNAP-25 for

synchronous transmitter release and in the regulation of spontaneous secretion but also suggested a role in vesicle priming (Weber et al., 2010).

2.3.2.2.3 Genetic analyses targeting syntaxin:

Syntaxin mutant alleles of Drosophila showed severe secretory defects consistent with a crucial role for syntaxin in both spontaneous and evoked transmitter release (Schulze et al., 1995). Although embryonic neuromuscular synapses in syntaxin null mutants exhibited normal responses to applied glutamate, spontaneous release events were undetectable and nerve impulses triggered rare, asynchronous quantal events. These results pointed to a critical role for syntaxin in regulated exocytosis at nerve terminals (Schulze et al., 1995). Subsequently, Wu and colleagues (1999) selectively mutagenized regions of syntaxin implicated in interactions with other presynaptic proteins, such as the Drosophila equivalent of unc-18 (known as, rop), and presynaptic Ca^{2+} channels. In both instances, increases in evoked transmitter release were observed, suggesting that these regions normally mediate interactions that constrain vesicular exocytosis.

In nematode worms, the unc-64 locus encodes syntaxin, and although there is appreciable similarity in the phenotype of Drosophila syntaxin mutants and unc-64 mutants, in general the worms appear less severely affected (Ogawa et al., 1998; Saifee et al., 1998). However, in an interesting test of the importance of the interaction of syntaxin and the unc-13 gene product, Richmond and co-workers (2001) reported that mutations producing an “open” form of syntaxin (in which the N-end of syntaxin no longer binds to its SNARE motif) bypassed the need for unc-13 in synaptic vesicle priming. More recently, a further analysis of synaptic vesicle docking in C. elegans led to the conclusion that syntaxin was essential for the docking process (Hammarlund et al., 2007).

Although mouse mutants of the syntaxin 1a gene have been generated, there remain several unresolved issues. The initial work (Fujiwara et al., 2006) targeted exons of syntaxin that could

have led to the expression of a truncated form of syntaxin (McRory et al., 2008). Presence of truncated syntaxin 1a, or genetic compensation may have contributed to the viability and normal properties of synaptic transmission observed in these KO mice. Using a different KO strategy, a very small number of syntaxin 1a KO offspring were recovered (McRory et al., 2008). However, it has not been established whether these survivors were due to the compensatory expression of another syntaxin gene. Thus, additional work will be needed to clarify the importance of syntaxin 1a for the function of vertebrate synapses.

2.3.2.3 Acute perturbation studies of SNARE protein function:

Among the seminal studies of SNARE function at the synapse, Hunt and colleagues (1994) monitored the impact on evoked transmitter release of presynaptic introduction of clostridial neurotoxins known to target synaptobrevin. The toxins caused a slow blockade of evoked transmitter release at the squid giant synapse that was largely complete after 2-3 h (similar results were seen at Aplysia synapses; Schiavo et al., 1992). However, tetanus toxin did not affect stimulus-dependent increases in presynaptic cytosolic Ca^{2+}, nor did it diminish the number of docked synaptic vesicles. These data implied that the toxin was affecting a post-docking step in the secretory cascade. Additionally, these authors found that a soluble, recombinant form of synaptobrevin reversibly blunted stimulus-dependent transmitter release in this system. These data clearly pointed to an important role for synaptobrevin in synchronous, evoked transmitter release.

Acute perturbation of syntaxin function has been reported by several groups. Mochida and colleagues (1995) used botulinum toxin type C as well as syntaxin antibody to inhibit evoked transmitter release at synapses formed by sympathetic neurons in culture. O'Connor and colleagues (1997) injected botulinum toxin type C or the recombinant H3 domain of syntaxin into the squid giant synapse and obtained a gradual, irreversible inhibition of evoked responses with the former and a reversible, ~60% reduction of stimulus-dependent secretion with the latter.

Similar results were reported for the type C toxin by Marsal and co-workers (1997). And, Sugimori and colleagues (1998) injected antibody targeting squid syntaxin into presynaptic elements of the squid giant synapses and observed a gradual block of stimulus-evoked transmitter release without any effect on the presynaptic Ca^{2+} current. Finally, infusion of H3-domain peptide into hippocampal neurons reduces evoked synaptic responses (Mishima et al., 2002). Collectively, these results support a key role for syntaxin in evoked neurotransmitter release.

SNAP-25 function was studied in lamprey by presynaptic injection of SNAP-25 antibody (Low et al., 1999). Stimulus-dependent responses were eliminated within minutes of antibody infusion without affecting currents through presynaptic Ca^{2+} channels, or the morphological appearance of the synaptic vesicle pool (Low et al., 1999). Again, these results are consistent with a crucial role for SNAP-25 in evoked neurotransmission.

2.3.2.4 SNARE-mediated membrane fusion in vitro:

In such classical examples as glycolysis and the Krebs cycle, biochemists have managed to reconstitute increasingly complex cellular processes. The obvious benefit of such efforts is that once a reaction sequence is reproduced *in vitro*, one can probe in greater detail the function of individual components of the system. And, this was the promise inherent in the demonstration by Weber and colleagues (1998) that SNARE proteins enhanced the rate of lipid mixing when they were incorporated into liposomes, *in vitro*. This finding was interpreted as showing that SNARE proteins constituted the “minimal machinery” for driving biological membrane fusion (Weber et al., 1998). Since then, a multitude of *in vitro* reconstitution experiments has generally supported this claim. However, in addition to the fact that under suitable conditions lipid bilayer membranes can fuse without any added protein (see section 2.1.2), there are many reasons to be skeptical of the relevance of these results to Ca^{2+}-dependent, synchronous transmitter release at nerve endings. The following discussion enumerates these concerns. In addition,

constructive suggestions will be offered for reconstitution experiments that may be better suited for illuminating mechanisms of fast, synaptic vesicle exocytosis.

Concern #1: Experiments need to measure membrane fusion, not lipid mixing: Over the last several years, there has been increasing awareness that commonly used *in vitro* membrane fusion assays report the extent of lipid mixing rather than membrane fusion (Dennison et al., 2006; Wang et al., 2009; Chan et al., 2009; Ohya et al., 2009; Kyoung et al., 2011, 2013; Diao et al., 2013). Although lipid mixing may occur prior to exocytosis at nerve terminals, it is the discharge of the contents of synaptic vesicles that underlies fast, chemical signaling at synapses. Thus, for reconstitution experiments to be relevant to fast, synaptic vesicle exocytosis, they need to measure fusion. Moreover, they need to be able to distinguish fusion from vesicle lysis. Increasingly robust procedures for monitoring bona fide membrane fusion *in vitro* continue to be developed and refined (Wang et al., 2009; Chan et al., 2009; Ohya et al., 2009; Rawle et al., 2011; Kyoung et al., 2011, 2013; Diao et al., 2013; Otterstrom and van Oijen, 2013), and their use will be important for continuing advances in this arena.

Concern #2: Synaptic vesicle exocytosis can occur in 0.2 msec or less, while the fastest *in vitro* SNARE-driven membrane fusion events occur <u>much</u> more slowly. This is the Achilles heel of every SNARE-based, lipid mixing or membrane fusion study published to date. The majority of investigations have reported fusion reactions with time courses of seconds to minutes, and even hours (Nickel et al., 1999; Parlati et al., 1999; McNew et al., 1999, 2000; Mahal et al., 2002; Melia et al., 2002; Tucker et al., 2004; Schuette et al., 2004; Bowen et al., 2004; Chen et al., 2006; Dennison et al., 2006; Pobbati et al., 2006; Chicka et al., 2008; Liu et al., 2008; Cypionka et al., 2009; Ji et al., 2010; van den Boogart et al., 2010; Lee et al., 2011; Christensen et al., 2011; Kim et al., 2012; Shi et al., 2012). However, systematic efforts have also been made to engineer reconstitution systems capable of detecting faster events. For instance, using supported bilayers in a TIRF-based system, reconstituted fusion events were reported to require

<100 msec (Fix et al., 2004). Subsequently, Liu and colleagues (2005; 2008) observed SNARE-driven fusion times of 25 msec. More recently, fusion times in the 5-10 msec range were reported for the interaction of liposomally bound synaptobrevin with supported bilayers containing t-SNAREs (Wang et al., 2009). Using a similar system, Domanska and colleagues (2009; 2010) also observed fusion kinetics in the 5-10 msec range. However, slower fusion times (~130 msec) have also been reported for supported bilayer systems (Karatekin et al., 2010). In the last couple years, a versatile, single-vesicle, content-mixing assay gave fusion times <100 msec (Kyoung et al., 2011, 2013; Diao et al., 2012, 2013). However, these fusion times are all at least one order of magnitude slower, and typically 2-4 orders of magnitude slower than exocytotic fusion at nerve terminals. Although this temporal discrepancy has been noted in other commentaries (Duman and Forte, 2003; Kiessling, 2005; Brunger, 2006), and may in part be due to technical limitations of the *in vitro* assay systems (Brunger et al., 2009; Otterstrom and van Oijen, 2013), this disparity remains a major stumbling block for efforts to relate *in vitro* fusion data to the *in vivo* system. Objectively, these data indicate that SNAREs cannot mediate membrane fusion for fast exocytosis at nerve terminals. This conclusion reflects the fact that the fusion events that SNAREs catalyze *in vitro* are prohibitively slower than what is observed at functional synapses. In this context, it is important to consider the possibility that the assay systems used to date lack one or more molecular components that are critical for catalyzing the type of sub-millisecond exocytosis that is detected *in vivo*. As an analogy, if one wanted to reconstitute the rapid conduction of action potentials observed in many vertebrate axons *in vivo*, one would find that myelination was essential. Without myelin, axonal conduction would remain slow. By the same token, *in vitro* fusion systems clearly lack one or more components needed for kinetic alacrity. Consequently, until *in vitro* assays achieve fusion kinetics similar to nerve terminals, their relevance to the biological situation will remain suspect.

Concern #3: We currently know little about the post-translational status of the proteins that participate in fast exocytosis at nerve terminals, and even less is known about the functional

consequences of these modifications. Most *in vitro* reconstitution experiments have used bacterially produced recombinant proteins. Typically, these bacterial products will not be post-translationally processed in the same manner that they would be in eukaryotic cells. For instance, synaptotagmin I and synaptobrevin 2 are palmitoylated (Veit et al., 1996; Chapman et al., 1996; Veit et at., 2000), and bacterially expressed forms of these proteins will lack this alteration. Moreover, recent work by Vrljic and colleagues (2011) revealed that synaptotagmin I produced in insect cells possessed a number of unexpected post-translational modifications, including nitration of tyrosine residues. Clearly, further work will be needed to assess the functional significance of these covalent alterations. This concern will be reprised in a later discussion (section 2.7) in which specific functional contributions are proposed for the palmitoylated cysteine residues of synaptotagmin I.

Concern #4: The membrane density (per unit area) of SNARE proteins in synaptic vesicles and at active zones is not known with confidence: The original studies of SNARE-mediated lipid mixing used very high protein:lipid ratios of ~1:100 (Weber et al., 1998). These ratios subsequently were recognized as being non-physiological, and fusion assays using lower protein:lipid ratios led to equivocal results concerning the role of SNAREs as mediators of membrane fusion (Bowen et al., 2004; Dennison et al., 2006). However, many reconstitution studies have since relied on evidence that individual synaptic vesicles harbor ~70 copies of synaptobrevin 2 (Takamori et al., 2006). This value must be regarded as an upper limit, because Mutch and colleagues (2011) independently reported an “average” value of 10.5 copies per vesicle of synaptobrevin 2 with almost half of the vesicles they analyzed harboring 8 or fewer copies of this v-SNARE. Nevertheless, these values indicate that synaptobrevin 2 is present on synaptic vesicles at a density of 5,000 to 50,000 per μm^2, which is in the same range reported for acetylcholine receptors at the neuromuscular junction or sodium channels at nodes of Ranvier. At the same time, information is not presently available concerning the membrane density of SNAP-25 and syntaxin 1a at nerve terminal active zones. Clearly, additional work will

be needed to establish the biologically relevant range of SNARE densities (per unit area of membrane) for use in fusion investigations, *in vitro*.

Concern #5: Most *in vitro* reconstitution experiments can be interpreted as indicating that the main role of SNAREs is to bring membranes into close proximity: This assertion is diametrically opposed to the conclusion drawn in an early study in which it was alleged that SNAREs do more to promote fusion than bring membranes close together (McNew et al., 2000). Because of the importance of this issue for understanding the role of SNAREs, it is crucial to review the data supporting the view that the major role of SNAREs is to facilitate membrane proximity/contact.

First, it is salient that the lipid tethers that replaced the SNARE transmembrane domains in the work of McNew and co-workers (2000) did not discernibly alter the initial kinetics of lipid mixing (their Fig.2A), while at two hours, lipid mixing was ~15% of the level achieved with standard, recombinant SNAREs. The fact that lipid mixing events were not completely blocked implies that membrane proximity was still a factor in these interactions. Thus, contrary to the inferences of McNew and colleagues (2000), the outcome of these experiments did not unambiguously demonstrate that SNAREs do more than facilitate membrane contact.

A second finding that points to the importance of proximity emerged from the work of Bowen and coworkers (2004) who found that v-SNARE liposomes could be induced thermally to fuse with t-SNARE-containing membranes lacking SNAP-25 (similar observations were reported by Liu et al., 2005). These results indicated that the appreciably weaker interaction between synaptobrevin and syntaxin was adequate to drive fusion in this system and that the full SNARE complex was dispensable. Phrased differently, one could conclude that membrane proximity mediated by weak interaction between v- and t-SNAREs suffices for fusion, *in vitro*. In a similar vein, it was recently reported that the formation of just one SNARE complex promoted liposome fusion *in vitro* (van den Boogart et al., 2010; Kiessling et al., 2010). In these latter experiments, it is clear how a single SNARE complex could enhance membrane proximity and subsequent,

thermally driven membrane fusion. However, it is less evident how this result fits with the SNARE-mediated fusion scenarios in Fig.3 which invoke a minimum of two SNARE complexes.

Nevertheless, the data providing the strongest support for the idea that proximity is a necessary and sufficient factor in mediating membrane fusion *in vitro* comes from experiments using a variety of “SNARE surrogates”. For instance, Richard and colleagues (2004) used the binding of metal ligands to synthetic lipoligands to induce liposome fusion, *in vitro*. This fusion reaction was sensitive both to the concentration of incorporated lipoligand and to the dimensions of the lipoligand molecule. Similarly, Haluska and co-workers (2006) produced amphiphilic, β-diketone ligands which triggered the europium-dependent fusion of giant, unilamellar vesicles. But, the most versatile systems have used membrane anchored DNA (Stengel et al., 2007; Chan et al., 2009; Lygina et al., 2011). In these systems, complementary strands of DNA mounted on separate populations of liposomes were found to induce membrane fusion. For the purposes of the current argument, the most important observation was that increasing the length of membrane-proximal, non-complementary DNA linkers led to a decline in lipid mixing and content mixing (Chan et al., 2009). In other words, linkers that did not facilitate close contact between opposed liposomes were less effective at promoting membrane fusion. The unavoidable conclusion from these results is that proximity facilitates membrane fusion, *in vitro*. Thus, molecular devices that encourage membrane contact also promote membrane fusion. SNAREs are exquisitely designed to promote membrane contact. However, to date, they have not convincingly been shown to mediate any other “fusion-promoting” activity.

Of course, the role of membrane proximity is likely to be appreciably more complicated, *in vivo*, where a large array of proteins at nerve terminals adds layers of regulation to the situation being modeled, *in vitro*. Possibly the best example of this added complexity comes from experiments which assessed the functional impact of mutated versions of synaptobrevin 2 with 12 or 24 amino acid residue insertions between the SNARE motif and the transmembrane domain (Deak

et al., 2006 and see section 2.3.2.2.1). The 12 residue insertion restored spontaneous transmitter release to the WT level, but stimulus-evoked responses only recovered from the KO level of ~8% up to ~25% of the WT control. By comparison, the 24 residue insertion did not rescue spontaneous events and evoked responses barely reached ~20% of the WT control. These findings indicate that structural changes that are likely to alter the "tightness" of SNARE bundling, and presumably alter vesicle-plasma membrane proximity have variable effects on spontaneous and regulated exocytosis. However, appreciably more work will be needed to clarify the underlying mechanism of these distinctive functional outcomes.

Concern #6: The majority of *in vitro* membrane fusion assays do not attempt to reconstitute the Ca^{2+} dependence of membrane fusion events: Clearly, many *in vitro* assays are undertaken to assess the role either of SNAREs themselves or other regulatory molecules in the membrane fusion process. Hence, the type of Ca^{2+}-dependence that is germane to synchronous transmitter release *in vivo* may be a secondary concern. Nevertheless, because stimulus-dependent, synaptic vesicle exocytosis is Ca^{2+} dependent, ultimately it will be necessary to clarify the role of synaptotagmin in conferring Ca^{2+}-dependence to *in vitro* fusion reconstitutions. Recent investigations which have used systems with better temporal resolution and/or improved fusion assays (Lee et al., 2010; Kim et al., 2012; Diao et al., 2012; 2013) augur well for ongoing efforts to clarify the role of Ca^{2+} and synaptotagmin in model membrane fusion events.

2.4 SNARE-based Models of Membrane Fusion: Variations on a Theme

The ensuing discussion addresses the distinctive features of the different SNARE-based models of membrane fusion in Fig.3. Because these models make subtly different structural and functional predictions, this section will point to where future technological advances should help to distinguish among these models. This section closes by addressing evidence regarding the number of SNARE complexes needed to drive membrane fusion.

2.4.1 The annulus model:

Annulus models are based on the idea that release-ready synaptic vesicles (defined in section 2.5) make physical contact with the plasma membrane. SNARE complexes are then arrayed in a ring around this contact area (see Pantano and Montecucco, 2013 for a recent example of an annulus scheme). To trigger exocytosis, synaptotagmin is presumed to initiate a reaction that is transmitted via the SNAREs to the lipid interface between the vesicle and the plasma membrane thereby leading to fusion presumably via stalk/hemi-fusion intermediates. Thus, the two distinctive features of this model are the circumferential organization of the SNAREs and the fact that SNARE complex formation occurs (or, is completed) after the vesicle has contacted the plasma membrane.

The major issue with annulus models is that they do not explain how the ring of SNAREs perturbs the lipids within the ring to cause fusion. Clearly, it makes sense intuitively that the energy released by SNARE-dependent interactions could clamp the vesicle more tightly to the plasma membrane and possibly lead to lipid re-arrangements compatible with fusion. However, one could as easily argue that this reaction promotes inward or outward bulging of the vesicle-plasma membrane interface or even vesicle lysis. Indeed, Heuser (1976) presented electron microscopic evidence that synaptic vesicles can induce large concavities in the plasma membrane without causing membrane fusion. Or, if SNARE bundling is not suitably coordinated around the vesicle periphery, the vesicle might just wobble. In summary, in spite of a relatively lengthy history, the field has not converged on a reliable explanation of how SNARE protein interactions influence membrane lipids in a direction compatible with fusion. For an event as important as synaptic vesicle exocytosis, it is unlikely that a pathway with such a range of potentially unproductive side reactions would have survived. Regardless, should future data emerge in support of annulus models, it will still be necessary to delineate how SNARE bundling is coordinated around the vesicle periphery and how the energy released by SNARE bundling conveys a signal to the lipids within the vesicle-plasma membrane contact area to promote the transitions (stalk/hemi-fusion) that lead to fusion.

The second criticism of annulus models is that they need to invoke a mechanism to prevent SNARE zippering or to achieve an intermediate arrest of SNARE zippering. This requirement exists, because when vesicle-associated synaptobrevin approaches sufficiently closely to the t-SNAREs (which is unavoidable, once a vesicle contacts the plasma membrane), one needs a mechanism to restrain synaptobrevin from interacting with the t-SNAREs. Although numerous different macromolecules, including synaptotagmin, complexin, munc-18, munc-13 or combinations of these proteins, have been proposed to fulfill this “clamping” or “partial arrest” role, there currently is no consensus regarding the underlying molecular mechanism. In fact, a recent review (Jahn and Fasshauer, 2012) noted several problems with this scenario. Among the unresolved issues are the experimental difficulty of capturing trans-SNARE complexes, the meager evidence for synaptotagmin/complexin regulation of SNARE assembly and the challenge of reconciling the large free-energy change accompanying SNARE complex assembly with the idea that this reaction can be arrested at an intermediate stage (ie., partial zippering of the SNARE complex). Clearly, additional work will be needed to shed light on these concerns.

The preceding discussion touched on another issue that is central to all models of synaptic vesicle exocytosis: namely, what is the morphological status of a “release ready” synaptic vesicle? As noted previously, the vast majority of electron micrographs of nerve terminals show synaptic vesicles which appear to be in direct contact with the plasma membrane (abundant examples can be seen in: Gray and Guillery, 1966; Peters et al., 1970; Pappas and Purpura, 1972). However, recent investigations in which the use of chemical fixatives was avoided reported that membrane-proximal synaptic vesicles were typically connected to the plasma membrane via short tethers (<5 nm), while the number of vesicles in direct contact with the plasma membrane was very low (Fernandez-Busnadiego et al., 2010; 2013). These observations raise a crucial question: “Do release-ready synaptic vesicles normally reside several nm from the plasma membrane?” The answer to this question will profoundly influence our understanding of the exocytotic process. If the answer is yes, then annulus models (at least,

as defined here) are excluded. Instead, such data would favor the hover model, but would not exclude the nipple or protein pore models (Fig.3). Because this issue of the morphological status of release-ready vesicles is so important, it will be considered separately in section 2.5.

2.4.2 The nipple model:

The difference between the nipple model and the annulus model is that the synaptic vesicle now sits on top of a thin, protein pedestal formed by partially zippered SNARE complexes (Fig.3B). Concurrently, the membrane spanning domains of synaptobrevin and syntaxin encircle a central disc of membrane lipids. Once the signal for membrane fusion is received, the SNAREs finish zippering and presumably induce a bulge in the central lipid disc which culminates in a stalk intermediate that leads to membrane fusion. Nipple models are essentially variations on the "SNAREpin" hypothesis originally advanced by Weber and colleagues (1998).

The nipple model predicts that release-ready synaptic vesicles will be separated from the plasma membrane by the minimum distance needed to accommodate the proposed arrangement of SNAREs (Fig.3B). Because structural studies indicate that SNARE coiling can continue through the linker regions of synaptobrevin 2 and syntaxin 1a to reach their respective transmembrane domains (Stein et al., 2009), the minimum separation between the vesicle and plasma membrane will be equivalent to the cross-section of a protein α-helix (~1 nm). Of course, this ~1 nm separation depends on the helical coils lying side-by-side rather than being stacked. This minimum separation also will be influenced by the presence of regulatory proteins (eg., complexin, synaptotagmin, munc-18) which could cause the vesicle-plasma membrane separation to exceed the 3-4 nm cross-section of the core SNARE complex. Thus, for practical purposes, nipple models should be discernible from annulus models based on the distance separating the vesicular and plasma membranes for "release-ready" vesicles.

Of course, nipple models still are subject to most of the criticisms leveled at annulus models. For instance, it has not been clearly explicated how or why SNARE zippering leads to nipple

formation; nor, is it necessarily clear why the nipple should protrude toward the plasma membrane versus the vesicle interior. In addition, the concerns of Jahn and Fasshauer (2012) noted above for the annulus model also pertain to the nipple models. Nevertheless, as with annulus models, the field is on the threshold of acquiring data that should weigh decisively in favor of or against nipple models (see section 2.5).

2.4.3 The hover model:

This model was developed to overcome the concerns enunciated by Jahn and Fasshauer (2012) in assessing other SNARE-based fusion models. Briefly, it avoids the problem of arresting SNARE bundling because it situates release-ready synaptic vesicles at a sufficient distance from the plasma membrane that SNARE-complex formation cannot commence. Synaptotagmin is proposed to play a key role in this scenario by serving as a "distance regulator", basically a protein platform that prevents the vesicle from making close contact with the plasma membrane (van den Boogart et al., 2011). The exocytotic cascade is then triggered by removing the synaptotagmin barrier, thereby allowing the vesicle into range for SNAREs to engage and drive membrane fusion.

The hover model has three attractive features. Obviously, it circumvents the criticisms Jahn and Fasshauer (2012) directed at annulus and nipple models. Second, it is consistent with the images showing that synaptic vesicles appear to be tethered at variable distances from the plasma membrane, but are seldom in direct contact (Fernandez-Busnadiego et al., 2010; 2013). Third, it includes a clear role for synaptotagmin, and it implies that synaptic vesicle distribution at nerve terminals should be altered in synaptotagmin KO mice. However, it also creates a separate set of problems. First, it requires a mechanism to keep the "distance-regulating" synaptotagmin(s) on the vesicle pole that is pointing toward the plasma membrane. Second, synaptobrevin(s) also need to remain poised on this pole to ensure that they can efficiently engage t-SNAREs. Third, this model requires there to be a transport step to move the synaptic

vesicle sufficiently close to the plasma membrane for fusion to occur (and/or a mechanism to invaginate the plasma membrane to meet the vesicle). This third problem was addressed previously (in section 2.1.1) with the conclusion that it will be necessary to demonstrate that the requisite translocation of the synaptic vesicle or the dimpling of the plasma membrane can be achieved in the fraction of a millisecond available for fast, synaptic vesicle exocytosis. Of course, it can be argued that the combination of the binding of synaptotagmin's C2 domains to the plasma membrane, along with SNARE coiling may provide the requisite energy, but it still will be necessary to verify that these events can transpire within 60 μsec. Fourth, this model is the most vulnerable of the four models to concerns about interference from other proteins affiliated with the vesicular and plasma membranes. In other words, the several nm of separation between a synaptic vesicle and the plasma membrane presumably allows proteins on both membrane surfaces free access to the interface that is destined to fuse. Because the intrusion of other proteins (both from the cytosol and associated with the surface of the opposed membranes) into this target zone is very likely to impede fusion, mechanisms must be found to prevent interference by extraneous proteins. Fifth, this model also does not explain what SNAREs do to membrane lipids to induce membranes to fuse. Thus, this scenario creates more problems than it solves.

2.4.4 The proteinaceous pore model:

This model is an extreme version of the nipple model in that the transmembrane domains of synaptobrevin and syntaxin form a ring at the point of vesicle-plasma membrane contact (Fig.3D). Once fusion triggering is initiated, the completion of SNARE bundling is proposed to lead to a lateral tilting of the membrane-spanning domains of each SNARE complex in a fashion that promotes the formation of a fusion pore and exocytotic membrane fusion (Jackson, 2010).

Morphologically, like the nipple model, this model requires the same narrow gap (~1-4 nm to accommodate the partially zippered SNARE complexes) between a release-ready vesicle and

the plasma membrane. However, if/when SNAREs can be identified in freeze-fracture replicas, this version demands a tighter packing of the SNARE transmembrane domains than the nipple model. This issue of the different morphological signatures of SNARE models will be reprised in section 2.5.

The most attractive feature of this model is that it provides a pathway by which the vesicle-plasma membrane interface can be perturbed in a direction that will lead to membrane fusion. Thus, it is the only SNARE-based model that explicitly addresses the mechanism by which SNAREs can elicit membrane fusion (see Jackson, 2010 for details). However, it is still subject to the same set of criticisms that Jahn and Fasshauer (2012) leveled at other SNARE models, and it does not offer any clarification of the role of synaptotagmin.

2.4.5 How many SNARE complexes are needed to drive membrane fusion?

While this topic continues to be debated (van den Boogart and Jahn, 2010; Pantano and Montecucco, 2013), three of the models in Fig.3 are compatible with as few as two SNARE complexes driving membrane fusion. At the same time, the proteinaceous pore model is based on the involvement of six SNARE complexes (Jackson, 2010). Recent empirical efforts suggest, in fact, that 2-3 SNARE complexes may suffice to enable fusion to occur (Mohrmann et al., 2010; Sinha et al., 2011). However, these assessments have been criticized by Pantano and Montecucco (2013), who proposed instead that a radial arrangement of 8 SNARE complexes underlies fast exocytosis at neuromuscular junctions. Once again, this is a matter that will be easier to resolve with improvements in the ultrastructural localization of proteins at active zones. It should also be apparent that whatever number of SNARE complexes ultimately is settled upon, it still will be necessary to clarify whether mechanisms exist to maintain a local population of t-SNAREs in the presynaptic membrane. Some insight into this issue has come from studies of lateral movements of syntaxin in the plasma membrane of cultured neurons (Ribrault et al., 2011), but it clearly will be important to extend this work to higher spatial resolution.

2.5 Can the release-ready pool of synaptic vesicles be defined morphologically?

SNARE-based models of exocytosis (Fig.3) make distinctive predictions about the location of synaptic vesicles and SNAREs for a “release ready” vesicle. This “release ready” pool is operationally defined as the subset of vesicles that is available for release in response to a single action potential. Practically, this pool is part of and typically only a small fraction of the “readily releasable” pool of vesicles defined by hypertonic sucrose (in other words, it is likely that heterogeneity of release probability exists within the readily releasable pool). Regardless, in the annulus model, a release-ready vesicle must contact the plasma membrane (the dimensions of this contact zone have not been established) with SNAREs arrayed at the edge of the contact zone. In the nipple model, SNAREs sit between the synaptic vesicle and the plasma membrane. Given the dimensions of the SNARE complex, this means that there must be a gap of 1-4 nm between the two membranes. The same is true for the protein pore model, only in this case the SNAREs should form a relatively tight bundle at the vesicle-plasma membrane interface. Finally, the hover model presumably requires a separation of the vesicular and plasma membranes of at least 4-5 nm with SNAREs being poised to zipper as soon as the membranes are brought into sufficient proximity to allow SNARE coiling. As discussed below, high resolution tomographic reconstructions of appropriately fixed and processed nerve terminals should help to distinguish among these models. Additionally, if technology for detecting SNARE proteins in freeze-fracture replicas can be achieved, the resulting data would also help to clarify which model in Fig.3 is correct.

The possibility that release-ready synaptic vesicles are poised sufficiently far from the plasma membrane to preclude SNARE bundling is supported by the work of Fernandez-Busnadiego and colleagues (2010) who reported that vesicle-plasma membrane contacts were very infrequent. Because this investigation used tomographic reconstruction of unstained, frozen specimens, it presumably avoided artifacts that can accompany the usual chemical fixation,

dehydration and heavy-metal staining of biological specimens for electron microscopy. If this approach turns out to be the new gold standard for assessing synaptic structure (Lucic et al., 2013) and if future observations support the results of Fernandez-Busnadiego and colleagues (2010), these findings will force a substantial revision of current views of nerve terminal secretory dynamics. Specifically, as noted above, if release ready synaptic vesicles are normally poised several nm from the plasma membrane, this physical separation provides a straightforward mechanism to prevent SNARE zippering until the appropriate moment. Nevertheless, this scenario creates other problems (see 2.4.3) that will need to be solved before the hover model (Fig. 3C) can be accepted. At the same time, several electron microscopic studies that have used cryo-fixation to avoid artifacts due to chemical fixatives have yielded data that are at variance with the findings of Fernandez-Busnadiego and colleagues (2010). For instance, in the original investigation of fast-frozen frog nerve terminals (Heuser and Reese, 1981), vesicle-plasma membrane contacts were evident in thin sections of freeze-substituted material. Similarly, Szule and colleagues (2012) asserted that there was no difference between frog nerve terminals subjected to conventional fixation and the fast-frozen specimens they studied. Direct vesicle-plasma membrane contacts were a common feature of their tomographic reconstructions (Szule et al., 2012). Similarly, projections and tomographic reconstructions of specimens subjected to high pressure freezing and cryosubstitution have also consistently showed vesicles contacting the plasma membrane (Rostaing et al., 2006; Weimer et al., 2006; Siksou et al., 2007). Finally, even though it was alleged that vesicle contacts with the plasma membrane were “rare”, data presented by Fernandez-Busnadiego and colleagues (2010, 2013) indicate that direct contacts were present. For instance, the reconstruction in Fig. 1B (Fernandez-Busnadiego et al., 2010) shows vesicles touching the plasma membrane and similar contacts are seen in Fig. 2B of Fernandez-Busnadiego and colleagues (2013). Moreover, their analyses of vesicle distribution in nerve terminals include a 0-45 nm bin (Fernandez-Busnadiego, et al. 2010, 2013) from which one is led to infer that vesicles showing

a 0 nm separation must be in contact with the plasma membrane. Nevertheless, with the data currently available, the question whether “release ready” synaptic vesicles make direct contact with the plasma membrane at active zones remains unsettled and will require further scrutiny.

Freeze-fracture analysis has considerable potential to provide independent insight into the status of the release-ready vesicle pool. If synaptobrevin and syntaxin can be identified in freeze-fracture replicas, the annulus model predicts that they will be present at the rim of the vesicle-plasma membrane contact area. In other words, they will form an oval or ring with a circumference dictated by the area of vesicle-plasma membrane contact. In contrast, the nipple and proteinaceous pore models predict that SNARE transmembrane domains will be embedded in the membrane area between the vesicle and plasma membrane. For the proteinaceous pore, a central cluster of SNAREs (the formal model proposes six SNARE complexes; Jackson, 2010) should be evident, while an intervening disc of lipids should separate the SNAREs in the case of the nipple model. Lastly, the hover model makes no explicit predictions concerning SNARE organization. However, freeze-fracture analysis could aid in resolving some of the other concerns regarding this hypothesis, such as mechanism(s) for establishing membrane domains that are free of potentially interfering integral membrane proteins.

An observation that attended several early, freeze-fracture studies of exocytosis in non-neuronal cells was that the region of secretory granule contact with the plasma membrane was largely devoid of particles (and, presumably, integral membrane proteins; see: Chi et al., 1976; Orci et al., 1977; Lawson et al., 1977). However, later work suggested that this clearing of particles was likely to be an artifact of glutaraldehyde fixation (Chandler and Heuser, 1980). Nevertheless, as was argued for the hover model, when a synaptic vesicle approaches the plasma membrane, if the apposed surfaces contain a high density of proteins that play no role in the fusion sequence, it is likely that a mechanism exists to clear these surfaces of irrelevant proteins prior to the formation of a release-ready state. The rationale for this argument is that intimate contact of the

lipids of apposed membrane hemibilayers is assumed to be critical for enabling membrane fusion (see Gingell and Ginsberg, 1978 for further discussion of this point). If extraneous proteins are present, they will interfere with this hemibilayer contact and suppress fusion. If this assumption is valid, it suggests that nerve terminal active zones should have particle-free (and protein-free) patches. Moreover, the size of these patches would be expected to vary among the different SNARE models in Fig.3. For the annulus model, the particle-free patch should correspond to the area of the annulus, because no integral membrane protein that projects mass into the cytosol should be able to access the zone of vesicle-plasma membrane contact. By the same token, the nipple model requires SNAREs to be lodged between the vesicle and plasma membrane, but the narrow gap between the vesicle and plasma membrane should exclude most other membrane proteins. Similarly, the proteinaceous pore model predicts a cluster of a half-dozen SNARE particles at the vesicle-plasma membrane contact, but again, few other proteins should be able to access this region. For the hover model, the prediction is less obvious, because particle clearing could occur at the time of or shortly before secretory triggering. Clearly, since these models point toward different particle distributions at active zones, careful analysis of high resolution images could aid in discriminating among these models.

An important sidebar to this discussion concerns sampling issues for electron microscopic studies. Currently, there is no accepted methodology to identify morphologically the pool of release-ready vesicles at a nerve terminal. Presumably, the number of quanta discharged in response to hypertonic sucrose should represent an upper limit to the size of this pool. In most circumstances, Rosenmund and Stevens (1996) pointed out that single, depolarizing stimuli led to the release of about 4% of the vesicle pool defined by hypertonic sucrose. Thus, as Rosenmund and Stevens (1996) speculated, if the readily releasable pool corresponds to “morphologically docked” vesicles, then release-ready vesicles are a small subset of these morphologically docked vesicles. Even if we ignore the possibility that morphologically docked

vesicles in electron micrographs may be artifacts of sample preparation (as suggested by Fernandez-Busnadiego, et al., 2010), this still means that only a small fraction of docked vesicles are likely to evince the signature arrangement of SNAREs discussed above. Nevertheless, by analyzing a sufficiently large number of sites of vesicle-plasma membrane contact, even a sub-population that may constitute just 4% of the total number of contacts should be detectable. By the same token, sampling issues must also be taken into account when addressing the number of synaptic vesicles (per nerve terminal or per unit area of membrane) that make contact with the plasma membrane. This sampling consideration may underlie some of the apparent discrepancies among diferent groups (as discussed above) with respect to the frequency of vesicle-plasma membrane contacts.

2.6 How does synaptotagmin trigger SNARE-dependent exocytosis?

As the ensuing discussion will make clear, synaptotagmins are remarkably versatile proteins endowed with myriad biological capabilities. Included in this repertoire are: synaptotagmin binds acidic phospholipids (Perin et al, 1990) and exhibits Ca^{2+}-dependent binding to liposomes that contain acidic phospholipids (Brose et al., 1992). This binding to bilayer membranes includes partial penetration of the proximal bilayer leaflet by synaptotagmin (Frazier et al., 2003). In addition, at sufficient densities, synaptotagmin can influence membrane bending (Martens et al., 2007). Synaptotagmin also self-associates and is frequently detected as dimers and tetramers (Perin et al., 1991). Further, it interacts with many other proteins in the presence or absence of Ca^{2+}, including syntaxins (Bennett et al., 1992; Chapman et al., 1995), SNAP-25 (Gerona et al., 2000), the SNARE complex (Davis et al., 1999), clathrin-AP2 (Zhang et al., 1994) and receptors for activated C kinases (Mochly-Rosen et al., 1992). These diverse capabilities have contributed to the challenge of clarifying how the binding of Ca^{2+} by synaptotagmin leads to synaptic vesicle exocytosis.

The recent review by Jahn and Fasshauer (2012) did an excellent job of summarizing current ideas regarding the triggering by synaptotagmin of SNARE-dependent membrane fusion. While the current commentary follows a similar trajectory, the reader is strongly encouraged to consult the Jahn-Fasshauer review for further discussion of this issue. With the exception of the hover model (Fig.3) most other SNARE-based fusion models have converged on the idea that SNARE complex formation is arrested at an intermediate state (incomplete zippering) prior to fusion. The role of the Ca^{2+}-bound state of synaptotagmin then becomes one either of diminishing the energy barrier that was preventing full SNARE zippering, or of removing a macromolecular impediment to full SNARE zippering (where the macromolecule(s) could be synaptotagmin itself, or complexin, munc-18 or munc-13). Ca^{2+}-liganded synaptotagmin presumably achieves one of these outcomes by: (i) dissociating from the SNARE complex to allow full zippering; (ii) binding to the SNARE complex and either promoting zippering directly, or by displacing the macromolecule that was preventing full zippering; (iii) binding to the plasma membrane in a manner that destabilizes the region adjacent to partially zippered SNAREs; (iv) inducing inward dimpling of the plasma membrane in a manner that facilitates fusion; (v) causing fusogenic cross-linking of the vesicle and plasma membrane.

These proposals are subject to a number of concerns that have not been resolved. First, Jahn and Fasshauer (2012) pointed out that in spite of considerable empirical support for the notion that SNARE zippering can be trapped at an intermediate state, several problems persist with this idea. For instance, there is meager evidence for synaptotagmin or complexin affecting SNARE assembly. Moreover, trans-SNARE complexes have seldom been captured experimentally. And, there are several reasons to be skeptical that any SNARE-interacting protein can reliably intervene in the highly exergonic zippering reaction. In addition to these general criticisms, specific models of synaptotagmin action were also critiqued by these authors. Models demanding membrane destabilization or bending were inconsistent with the biologically relevant levels of synaptotagmin found on vesicles and the relatively minor perturbation of

membrane structure accompanying synaptotagmin insertion into membranes. Collectively, these considerations led Jahn and Fasshauer (2012) to favor the hover model in which synaptotagmin restrains synaptic vesicles at a sufficient distance from the plasma membrane to preclude SNARE coiling (Fig.3C). Obstacles faced by this model were discussed earlier (2.4.3).

Two broad conclusions emerge from the preceding discourse. First, in spite of extensive investigation, there is little agreement in the field regarding the molecular mechanism by which synaptotagmin regulates SNARE-mediated membrane fusion. Second, the membrane fusion process itself remains an enigma. Although SNAREs are widely seen as playing a pivotal role (Fig.3), the mechanism by which they affect the lipid structure of the opposing membranes to catalyze fusion largely remains moot. In an effort to circumvent these pitfalls, a novel model of fast, Ca^{2+}-dependent exocytosis was recently proposed (Gundersen and Umbach, 2013). The next section provides a brief summary of this model along with its strengths and weaknesses vis a vis conventional, SNARE-based models.

2.7 The dyad model: synaptotagmin binds Ca^{2+} and catalyzes membrane fusion

The central thesis of the dyad model is that synaptotagmin I (or II) can serve as the exocytotic Ca^{2+} sensor and the macromolecular template for catalyzing membrane fusion during synchronous transmitter release (Gundersen and Umbach, 2013). This dual function role for synaptotagmin takes advantage of certain structural features of synaptotagmins I and II, including the palmitoylated cysteine region and the juxtamembrane polybasic motif (see Fig.4 and Gundersen and Umbach, 2013), that have been neglected in other fusion models. The dyad scheme also relies on a unique arrangement of synaptotagmins at the point of contact between a release-ready synaptic vesicle and the plasma membrane (Figure 4, 5A). Moreover, as far as the dyad model is concerned, all of the other proteins previously implicated as crucial components of the pre-exocytotic and exocytotic cascades (including, rabs, rabphilin, munc-13, munc-18, complexin and the SNAREs) serve principally to establish the release-ready state of

the synaptic vesicle. This separation of duties has several beneficial consequences: It eliminates the arguments about whether SNARE zippering can or even needs to be arrested prior to fusion (obviously, arrest is unnecessary). It obviates the need to explain how synaptotagmin regulates SNARE function (no regulation is required). Most importantly, it lets SNAREs off the hook; they no longer need to fuse membranes. Instead, their primary role is to bring membranes into close quarters after which synaptotagmin can do the rest. So, what is it that synaptotagmin does in this model?

The dyad model (see Gundersen and Umbach, 2013 for a full discussion) begins with two pairs of synaptotagmins poised at the interface between a synaptic vesicle and the plasma membrane (Figs.4 and 5A). This arrangement is made possible because there is a 12 residue region of predicted β-structure which immediately follows the trans-membrane domain of synaptotagmin I (and II). For vertebrate synaptotagmins I and II, this 12 residue segment includes multiple cysteine residues which are known to be palmitoylated (Veit et al., 1996; Chapman et al., 1996; Heindel et al., 2003). This short stretch of β-structure is postulated to have two major roles. First, it is presumed to “cross link” the vesicle and plasma membrane (as shown in Fig.4). This cross-linking is due to the projection of alternating hydrophobic moieties from this β-structure region into the opposed membrane hemibilayers. Second, it allows the hydrophilic regions (including, both C2 domains) of the four apical synaptotagmins to extend into the aqueous environment beyond the region of vesicle-plasma membrane contact (Fig.4). The synaptotagmins at this interface are represented as being paired (Fig.5A), because biochemical evidence supports the Ca^{2+}-independent dimerization of synaptotagmins (Perin et al., 1991a; von Poser et al., 2000; Fukuda et al., 2001). Nevertheless, this model remains viable even if the synaptotagmins operate autonomously (as outlined in section 2.9). Finally, this “release-ready” organization of the synaptotagmins is posited to reflect the concerted outcome of the actions of the other “pre-exocytotic” proteins (including, munc-13/18 and the SNAREs),

which contribute to and maintain the release-ready state of the synaptic vesicle (see 2.9 for further discussion).

The next stage of the dyad hypothesis is that the binding of Ca^{2+} to the C2 domains of the four apical synaptotagmins prompts the insertion into the plasma membrane of the C2 domains. This membrane insertion event is accompanied by a rapid, lateral translocation of both apical synaptotagmin pairs (as schematized in Fig.5A-B). As long as the net lateral translocation exceeds the ~5 nm thickness of a biological membrane, it will enable the membrane spanning domains of the synaptotagmins to pivot from their initial orientation perpendicular to the plasma membrane to the position shown in Fig.5C. At this stage (Fig.5C), the membrane spanning domains of the synaptotagmins are parallel to the plane of the plasma membrane. This rotation of the transmembrane domains of the synaptotagmin pairs causes an outward protrusion of the synaptic vesicle membrane. Concurrently, the lateral movement of the membrane spanning domains of the synaptotagmins serves as a wedge to displace lipids of the inner hemibilayer of the plasma membrane thereby leading to an inward dimpling of the outer hemibilayer of the plasma membrane. Collectively, these protein movements and lipid re-arrangements create a nascent fusion pore whose lifetime and further dilation is governed by several variables. These variables include the lipid composition of the membranes, the length of time the C2 domains of the synaptotagmins remain embedded in the plasma membrane, the curvature stress on the vesicle membrane and the presence/action of other proteins that may regulate this process. Regardless, under normal circumstances, this sequence of events is postulated to lead to the exocytotic fusion of the vesicular and plasma membranes (Gundersen and Umbach, 2013).

The central assumptions of the dyad model are that: (i) fast membrane fusion requires a quartet of synaptotagmins at the vesicle-plasma membrane interface; (ii) the binding of Ca^{2+} by the C2 domains produces movements that lead to the lateral separation of this quartet of apical synaptotagmins; and (iii) that subsequent steps in the genesis of the fusion pore are primarily

driven by the need to shield synaptotagmin's hydrophobic surfaces from the aqueous environment (a general feature of all membrane fusion models: see Gingell and Ginsberg, 1978). At the same time, other variables that will influence whether full fusion occurs were noted above. How plausible are these assumptions?

The apical localization of the synaptotagmins (Fig.4 and 5A) is predicated on the idea that the 12 amino acid residues following the membrane-spanning α-helix can adopt β-structure. Although this prediction certainly needs to be tested empirically, for reasons discussed in greater detail (Gundersen and Umbach, 2013), it is difficult to envision any other secondary structure for this tract in vertebrate synaptotagmins I and II. This is because the covalent attachment of palmitic acid to the five cysteine residues in this region of mouse synaptotagmin I (Chapman et al., 1996; Veit et al., 1996; Heindel et al., 2003 or the seven cysteines in mouse synaptotagmin II) offers a limited number of options for shielding these fatty acyl groups within the membrane environment. In fact, there is no other secondary structure besides a β-strand that is compatible with the energetic necessity of keeping these fatty acyl moieties in a hydrophobic milieu. At the same time, it was also noted that this 12 residue sequence contains a pair of lysines (Gundersen and Umbach, 2013), and additional work will be needed to understand the role, if any, of these basic residues (for instance, do they interact with anionic counterparts or are they post-translationally modified?). Nevertheless, until evidence is presented to the contrary, it is reasonable to infer that this region of synaptotagmins I and II adopts β structure.

Even if one accepts that synaptotagmins can occupy the vesicle-plasma membrane interface, how do they get there? Again, this is an issue that will require empirical studies. However, it is noteworthy that Szule and colleagues (2012) documented a number of distinctive, sequential interactions that occur between a synaptic vesicle and the active zone material during a vesicle's progression to a morphologically docked state. These reactions presumably are

important for transitioning the vesicle to a “release ready” state. Hence, it is defensible to speculate that one of the steps in this pre-exocytotic phase is to move four synaptotagmins to the vesicle-plasma membrane interface. It was originally inferred that this movement of the synaptotagmins takes place after the vesicle contacts the plasma membrane, but it subsequently became apparent that the quartet of synaptotagmins could be arrayed at the contacting pole of the vesicle before it reaches the plasma membrane (Gundersen and Umbach, 2013). Indeed, a recent ultrastructural study suggests that synaptic vesicles have luminal structure that could help to organize proteins on the surface (Harlow et al., 2013). As efforts are made to distinguish experimentally between these explanations (pre- versus post-docking positioning of apical synaptotagmins), it is noteworthy that early biochemical experiments revealed that synaptotagmin tended to form dimers and tetramers (Perin et al., 1991a). The dyad model takes advantage of this tendency of synaptotagmin to form higher-order complexes (nevertheless, a similar model using monomers is outlined later).

Is it reasonable to think that the Ca^{2+}-triggered insertion of the C2 domains into the plasma membrane transmits enough force to cause the required lateral separation (of ~5 nm) of the transmembrane domains of the paired, apical synaptotagmins? This will obviously remain a key issue of contention for the dyad hypothesis, and at this time crucial information relevant to this matter is lacking. Most importantly, even with the proposed organization of the membrane spanning domains and β-strand motif of the synaptotagmins in Fig.4, it still is uncertain where their C2 domains will reside with respect to the plasma membrane. A factor in this consideration is that the primary sequences of synaptotagmins I and II include an abundance of basic residues immediately after the 12 residue β-strand region. It was suggested (Gundersen and Umbach, 2013) that these basic residues affiliate with the external surface of the release-ready synaptic vesicle in a manner that would allow the C2 domains to reside a mean distance of ~5 nm from the inner leaflet of the plasma membrane. Concomitantly, the intrinsically negative electrostatic potential of the C2 domains will tend to hinder their “resting” association with the

plasma membrane. But, with Ca^{2+} binding to the C2 domain, this electrostatic repulsion gives way to membrane insertion of the Ca^{2+}- liganded C2 domains. Hence, the C2 domains were presumed to translocate ~5 nm through the cytoplasm before inserting into the plasma membrane. This movement was also postulated to cause the polybasic linker region to dissociate from the synaptic vesicle and bind to the plasma membrane. Overall, these hydrophilic regions account for ~80% of the total mass of synaptotagmin I (or II). If ~80% of the mass of each synaptotagmin pair were to undergo the proposed directed movement, it seemed reasonable to infer that the remaining 20% of the protein might also move. The other way to approach this issue is to ask what restraints would be necessary to prevent 20% of the mass of each synaptotagmin dimer from moving while the other 80% underwent the ascribed translocation. Either way, empirical investigations will be necessary to resolve this matter.

Once the membrane-spanning domains of the apical synaptotagmins performed the rotational pivoting (as shown in Fig.5C), the dyad hypothesis suggested that full membrane fusion was likely to be driven principally by the curvature stress (bending energy) of the vesicle membrane. Again, this is a prediction that will require experimental study. Nevertheless, it is important to note that the dyad model is among the few theories of biological membrane fusion that provides an explicit mechanism for perturbing membrane structure at the vesicle-plasma membrane interface where fusion is slated to occur. This fact leads us to a more-systematic assessment of the advantages of the dyad model relative to SNARE-based fusion models.

2.8 Major advantages of the dyad model

2.8.1. *It is simple:* It avoids all of the complicated inter-molecular interactions that are typically present in SNARE-based models of membrane fusion. By having one molecular species serve both as Ca^{2+} sensor and catalyst of membrane fusion, one circumvents the raft of concerns enunciated for SNARE models. The only simpler scenario is one that avoids protein altogether.

2.8.2. *Fusion can be fast*: Regardless of the fusion model one prefers, synchronous exocytosis at nerve terminals presumably is initiated by the Ca^{2+}-dependent binding to the plasma membrane of synaptotagmin's C2 domains. In spite of uncertainty concerning the distance that the C2 domains have to travel to insert into the plasma membrane, this phase of the exocytotic cascade is unlikely to require more than 5 μsec (based on typical protein dynamics). Beyond this initial event, the dyad scenario has just two additional steps that lead to a fusion pore: the membrane dissociation and re-association of the polybasic region of each synaptotagmin pair, and the ~2.5 nm of lateral movement and pivoting of the transmembrane domain of each synaptotagmin. Although it remains to be determined how many of the basic residues in synaptotagmin's polybasic region interact electrostatically with the vesicle membrane, the dissociation-re-association steps should require no more than a few μsec. However, the lateral movement of the synaptotagmins will take longer. An upper limit on the elapsed time can be inferred from typical diffusion constants for membrane proteins. In the majority of cases, these values fall between 0.1-1 μm^2-sec^{-1} (Kusumi et al., 2005; Owen et al., 2009). Using this range of values, an upper limit for the time needed for a 2.5 nm lateral movement of synaptotagmin is ~20 μsec. Hence, the dyad model is compatible with exocytotic events that occur in <60 μsec.

2.8.3. *It explicitly targets a perturbation of membrane structure to the interface where fusion is destined to occur*: With the exception of the proteinaceous pore model (Fig.3D), most models of SNARE-dependent membrane fusion do not provide a step-by-step description of how and why SNARE zippering should lead to the fusion of apposed lipid bilayers [see reviews by Martens and McMahon (2008) and Sorensen (2009) for a further discussion of this void]. Instead, the dyad model intrinsically targets the synaptic vesicle-plasma membrane contact zone in a series of steps that plausibly lead to exocytotic membrane fusion. The proteinaceous pore model also makes explicit predictions regarding the development of a fusion pore, but it obviously uses different molecular machinery. Thus, information concerning the organization of synaptotagmin and SNAREs at the membrane interface between release-ready synaptic vesicles and the

plasma membrane will be vital for distinguishing between the fusion models of Fig.3 and the dyad scheme.

2.8.4. *It can explain the observed relationship between extracellular Ca^{2+} concentration and the number of quanta released in response to nerve stimulation*: In a quantitative analysis of the impact of extracellular Ca^{2+} on evoked transmitter release, Dodge and Rahamimoff (1967) reported a power relationship with an exponent of nearly four. Their interpretation of this result was that it typically required the binding of Ca^{2+} to approximately four sites at the nerve terminal to trigger the release of each quantal packet of transmitter. Although the interpretation of similar data obtained at other synapses has been mooted over the years (see Dittrich et al., 2013 for a recent discussion of this issue), it is noteworthy that the dyad model provides a straightforward molecular correlate of these results. Specifically, Ca^{2+} binding to the C2 domains of the four apical synaptotagmins (Figs.4, 5A) is proposed to be the event that triggers vesicular exocytosis. However, regardless of the model of exocytotic triggering that is ultimately proven to be correct, appreciably more work will be needed to clarify such issues as the relative contributions of the C2A and C2B domains to secretory triggering, the Ca^{2+}-binding affinity of these modules, and the fact that each C2 domain binds multiple Ca^{2+} ions.

2.8.5. *It makes specific empirical predictions*: For most other fusion models, the membrane spanning domain, the linker region and the polybasic region of synaptotagmin have no role other than targeting of synaptotagmin to synaptic vesicles and tethering of synaptotagmin to the vesicle surface. The dyad model ascribes specific functions to these regions. Thus, appropriately designed mutagenesis experiments (see Gundersen and Umbach, 2013 for examples) should help to distinguish the dyad model from the other hypotheses. Similarly, the dyad model predicts that high resolution microscopic experiments should reveal a quartet of synaptotagmins at the vesicle-plasma membrane interface. No other model makes this prediction. In this same context, the dyad model predicts that “release ready” synaptic vesicles

will be found in direct contact with the plasma membrane. This contrasts with the nipple, hover and proteinaceous pore models (Fig.3). Moreover, given the dimensions of the palmitoylated linker region of vertebrate synaptotagmins, one can estimate the dimensions of this contact region (~10 nm diameter; see Gundersen and Umbach, 2013 for discussion). Hence, systematic measurement of the vesicle-plasma membrane contact region will be an important index of the plausibility of the dyad scheme. Finally, with suitable attention to detail (including, the use of palmitoylated synaptotagmin), the dyad hypothesis anticipates that it should be possible to reconstitute Ca^{2+}-dependent, sub-millisecond membrane fusion of liposomes using synaptotagmin I or II alone. Although it is likely to be challenging to work with palmitoylated synaptotagmin in an *in vitro* system, the potential rewards should ultimately outweigh the drawbacks.

2.9 The dyad model: lingering questions, considerations and concerns

The dyad model was developed to provide a simple alternative to the cumbersome, SNARE-based models of synchronous transmitter release at nerve terminals. In addition to specific issues mentioned above (in sections 2.7 and 2.8), there are other matters that will need to be resolved:

Is it possible that the four synaptotagmins at the vesicle-plasma membrane interface (Fig. 4, 5A) operate autonomously? Yes. In fact, from the perspective of its underlying symmetry, this variation of the dyad model (shown schematically in Fig.6) is very attractive. It starts with four synaptotagmins poised as shown in Fig.5A, except that the synaptotagmins are not dimerized. Then, during fusion triggering, the synaptotagmins disperse radially from their central contact point (Fig.6A) to reach the stage in Fig.6B where their membrane spanning domains delimit a central pore. As with the dyad model, further dilation of this pore will be influenced by factors including membrane lipid composition, curvature stress on the vesicle membrane and other (as yet, unidentified) regulatory interactions.

Is the dyad model compatible with the variety of Ca^{2+}-dependent interactions that have been reported to occur between synaptotagmin and other macromolecules, or lipids, like PIP2? Invoking Occam's razor, the dyad model chose membrane penetration of synaptotagmin's C2 domains as the simplest mechanism to couple Ca^{2+} binding to the directional insertion of the hydrophilic, C-end of this protein into the plasma membrane. However, because of compelling evidence for the Ca^{2+}-dependent interaction of synaptotagmin with such plasma membrane proteins as SNAP-25 (Zhang et al., 2002) and lipids, like phosphatidylinositol bisphosphates (Bai et al., 2004; Radhakrishnan et al., 2009), it may emerge that the proposed binding of synaptotagmin to the plasma membrane also involves interaction with specific protein or lipid targets. Fundamentally, the presence of specific protein or lipid targets in the plasma membrane does not alter the motion-based mechanism by which synaptotagmin is proposed to induce membrane fusion. Clearly, this is an issue that reconstitution experiments are well suited to test.

Can anything be inferred from the findings that synaptic vesicles harbor 7-15 copies of synaptotagmin, while the dyad model requires only four synaptotagmins? An obvious possibility is that the additional synaptotagmins exist to ensure that enough copies are present for the dyad scheme to work. Another possibility is that the additional synaptotagmins are needed for endocytotic steps. Clearly, testing of this latter possibility will require finding a mechanism to alter the number of synaptotagmins per vesicle in a system where endocytosis can be monitored.

What is the function of the other synaptotagmin gene products, particularly the synaptotagmins that do not bind Ca^{2+}? This issue has remained perplexing ever since it was realized that the primary sequence of the C2 domains of some synaptotagmins included amino acid substitutions that preclude Ca^{2+} binding (Sudhof, 2002; Martens and McMahon, 2008). A recent study offered an interesting approach to this question. Hippocampal neurons were transfected with pHluorin conjugates of different synaptotagmin isoforms (Dean et al., 2012). The cells were then

examined to determine where the synaptotagmin-pHluorin conjugates were expressed as well as whether they showed discernible exocytotic and endocytotic cycling in response to high K^+ depolarization. The various synaptotagmin isoforms fell into four classes. Synaptotagmins I and II were the only synaptotagmins that associated with synaptic vesicles. Synaptotagmins III and XI preferentially localized to and recycled in dendrites. Synaptotagmins V, VII, X and XVII were restricted to axons, while synaptotagmins IV, VI, IX and XII were present in and recycled both in axons and dendrites. Interestingly, this investigation also took issue with prior work (Xu et al., 2007) in which it was postulated that synaptotagmin IX can localize to synaptic vesicles and restore hippocampal synaptic transmission in synaptotagmin I KO mice. Regardless, the upshot of this investigation is that it appears that synaptotagmins may contribute to a variety of membrane trafficking events in anatomically discrete regions of neurons (Dean et al., 2012). Nevertheless, the molecular role of synaptotagmin in these events remains to be clarified. In other words, it will be important to determine whether these synaptotagmins function primarily in the exocytotic or endocytotic arm of these membrane trafficking pathways.

Is the dyad model compatible with what is known about the function of munc-13 and munc-18? As noted previously (in 2.3.2.2), deletion of munc-13-1 and 13-2 or munc-18 genes leads to almost complete abolition of quantal transmitter release in mice, and it is reasonable to enquire how these munc proteins might be integrated into the dyad model. Currently, the most plausible scenario is that these munc proteins cooperate with SNAREs to establish the release-ready state of a synaptic vesicle. Precisely which reactions they mediate remains to be clarified. Nevertheless, because synaptic vesicles appear to undergo a sequence of macromolecular interactions as they approach the plasma membrane (see Szule et al., 2012 for discussion), and there is ultrastructural evidence that munc-13 contributes to the process by which vesicles reach the plasma membrane (Weimer et al., 2006; Gracheva et al., 2010), it is likely that the muncs operate at a step prior to when synaptotagmins are hypothesized to reach the vesicle-plasma membrane interface.

What is the relationship among the different modes of transmitter release at nerve terminals? The dyad model aims to explain the rapid coupling between Ca^{2+} influx and transmitter release at nerve terminals. However, nerve endings also exhibit spontaneous, quantal secretory events and asynchronous quantal secretion of the type that is triggered by hypertonic sucrose. Many of the investigations alluded to above have noted similarities and differences in the molecular regulation of these secretory events at nerve endings. An example that extends this theme is the apparent dichotomy between synaptotagmins II and VII in regulating synchronous and asynchronous transmitter release at the neuromuscular junction of zebrafish (Wen et al., 2010). Nevertheless, we still do not have a full picture of the underlying mechanism(s) that regulate(s) spontaneous secretion, nor has the basis of secretory triggering by hypertonic sucrose been elucidated. Clearly, additional work will be needed to resolve these issues.

Can the dyad model be used to explain secretory events in other cells that exhibit regulated exocytosis? Although the dyad scheme was developed to explain the rapid kinetics of synchronous transmitter release at nerve endings, this process certainly may operate in other cells as the final phase of the secretory cascade. For instance, interactions mediated by rabs, munc-13, munc-18 and/or SNAREs may be part of the secretory triggering process that precedes hormone secretion in adrenal chromaffin cells, pancreatic β-cells and other endocrine cells. However, it is also plausible that redundant or parallel secretory pathways operate independently of the dyad scheme (for example, see evidence for the role of rab-SNARE interactions in endosomal membrane trafficking; Ohya et al., 2009, and data indicating that α-latrotoxin promotes exocytosis via an unconventional pathway; Deak et al., 2009), possibly by exploiting one of the scenarios depicted in Fig.3. Further work will be needed to distinguish among these alternatives.

3. Conclusion

The nervous system is specialized for rapid communication, for information processing and for preserving memories of the recent and distant past. Synapses are widely regarded as being important for all of these processes. This review has focused on progress in understanding cellular and molecular events that underlie fast signaling by nerve terminals. Although there is little debate that presynaptic Ca^{2+} channels serve as crucial conduits for initiating the synchronous release of neurotransmitter at chemical synapses, abundant questions remain about the short- and long-term regulation of these channels. Similarly, synaptotagmins I and II are widely accepted as the proteins responsible for transducing Ca^{2+} signals at nerve endings. However, the mechanism by which synaptotagmins act remains controversial. Most models of synaptic vesicle exocytosis envision a role for synaptotagmins in regulating SNARE-mediated membrane fusion. A central theme of this review is that SNARE-based models of membrane fusion do not adequately explain the fast kinetics of exocytosis at nerve terminals. At the same time, a recent proposal was advanced in which synaptotagmins serve both as exocytotic Ca^{2+} sensors and as catalysts of membrane fusion. This latter model is compared and contrasted with scenarios that rely on SNAREs to fuse membranes. Investigations within the coming decade should help to distinguish among these competing hypotheses. However, it is likely to take appreciably longer before we unravel the molecular machinery by which synapses contribute to information processing, storage and retrieval.

Table 1: Presynaptic active zone Ca^{2+} channel types

Protein Name	Pharmacologic Name – blocker	Gene Name	Synprint site?
Cav 2.1 / α1A	P/Q - ω-Agatoxin IVA	Cacna1A	Yes
Cav 2.2 / α1B	N - ω-Conotoxin GVIA	Cacna1B	Yes
**Cav 2.3 / α1E*	*R - SNX-482*	*Cacna1E*	*No*

**Cav 2.3 type Ca^{2+} channels are only present as relatively minor contributors in a few types of synapses, and at other synapses under pathological conditions (Kamp et al., 2005; Pardo et al., 2006; Kaja et al., 2006). These channels do not appear to have a synaptic protein interaction (synprint) site, and may rely on C-terminus scaffold protein anchoring (Pereverzev et al., 2002).*

FIGURE LEGENDS

Figure 1. Diagram of voltage-gated calcium channel interactions with active zone proteins. A single synaptic vesicle release site is depicted with the major protein interactions that serve to bring Ca^{2+} channels in close proximity to docked synaptic vesicles. The limited number of locations that Ca^{2+} channels can occupy within an active zone are shown as oval "Ca^{2+} channel slots" – one of which is shown to include a Ca^{2+} channel. Laminin β2 binds directly to Ca^{2+} channels and may aid in active zone alignment with postsynaptic receptors (at least at the NMJ). The α2δ subunit of the Ca^{2+} channel is thought to bind within the synaptic cleft with unknown targets and may regulate Ca^{2+} channel density and positioning with active zones, while the SNARE proteins, synaptotagmin, RIM and RIM-BP bind directly to Ca^{2+} channels to aid in localizing these channels very close to docked synaptic vesicles.

Figure 2. Diagram of two ways to organize a single vesicle release site within an active zone. Left panel: Single vesicle release sites can be organized with very few Ca^{2+} channels that are each tightly coupled to docked synaptic vesicles. In this scenario, Ca^{2+} channels have a low probability of opening during a presynaptic action potential and the flux through a single open channel likely triggers synaptic vesicle fusion (nanodomain coupling). **Right panel:** Single vesicle release sites can alternatively be organized with a large collection of Ca^{2+} channels that are more loosely coupled to each docked synaptic vesicle. In this case, these Ca^{2+} channels often open with a higher probability during a presynaptic action potential, and the summed Ca^{2+} flux through many open channels provides the trigger for each synaptic vesicle fusion event.

Figure 3. Schematic depiction of the different hypotheses for explaining SNARE-mediated membrane fusion. Each image is a cross-section of a "release-ready" synaptic vesicle. Synaptobrevin (v-SNARE) is in dark blue; Syntaxin is in red and SNAP-25 is in green with its membrane-affiliated region shown as a dotted line. A. Annulus model: These models

rely on the evidence that synaptic vesicles make direct contact with the plasma membrane. SNARE proteins are thought to be arrayed around the zone of vesicle-plasma membrane contact, most likely in a partially zippered state. When synaptotagmin(s) bind(s) Ca^{2+}, full SNARE zippering occurs and the energy released presumably influences the lipids within the annulus in a direction leading to exocytotic fusion. Major questions that this scheme leaves unanswered are how SNARE zippering is arrested; how this arrest is relieved; how many SNAREs participate; how big the annulus is (its circumference); and what synaptotagmins do to trigger exocytosis. B. Nipple model: These models embed SNAREs very close to the pole of the synaptic vesicle that faces the plasma membrane. A small patch of membrane lipid separates the membrane spanning domains of the synaptobrevin molecules, while a similar patch of lipids separates the membrane-spanning domains of the syntaxins. The major difference between the nipple model and the annulus model is that the zipper-arrested SNAREs are largely situated between the vesicle and plasma membrane. With activation of synaptotagmin and full SNARE zippering, the membrane spanning domains of synaptobrevin and syntaxin are presumed to "pinch" or distort the membrane lipids between them to cause a stalk to form as a prelude to membrane fusion. The schematic here shows a nipple at an early phase of stalk formation. The major unanswered questions for these hypotheses are essentially the same as for annulus models. However, plausible explanations for the mechanism of nipple formation are lacking, and there is no good reason why the nipple is expected to evert rather than invert. C. Hover model: This model solves the problem of arresting SNARE coiling by fettering synaptic vesicles at a sufficient distance from the plasma membrane that v- and t-SNAREs cannot zipper. Synaptotagmin may play a role in maintaining this vesicle-plasma membrane separation, but once exocytotic triggering is initiated, SNARE coiling presumably brings the vesicle into contact with the plasma membrane and promotes fusion. The two major concerns with this model include: the need to demonstrate that the relatively large distance between the vesicle and plasma membrane (>4 nm) can be covered in the meager time available for exocytotic events at

nerve terminals, and its failure to explain what SNAREs do to induce membrane fusion. D. Proteinaceous pore model: Here, a ring of zipper-arrested SNAREs forms at the vesicle-plasma membrane interface. Once synaptotagmin acts, the SNAREs bend and move laterally to drive the formation of a proteolipid fusion pore. This model also needs a mechanism to arrest SNARE zippering and does not provide a satisfying explanation for how synaptotagmin triggers exocytosis.

Figure 4. Structural predictions of the dyad model. Proposed organization of synaptotagmins at the synaptic vesicle-plasma membrane interface: This schematic shows a cross-section of the vesicle-plasma membrane interface in a view that is expanded to reveal the cysteine-rich linker region and the vesicle membrane-associated polybasic region which are crucial for enabling the pair of mouse synaptotagmin I molecules to project their hydrophilic domains (linkers and C2A and C2B domains) into the cytosolic space adjacent to the vesicle. The cysteine-rich linker region is expected to adopt β-structure. The cysteines of this region are fatty acylated (shown as squiggly lines), while the other large hydrophobic residues are shown with ovals. The tandem Lysyl residues of the linker have + signs. The transmembrane (TM) domain of each synaptotagmin is a large, vertical oval with a kinked line representing the intraluminal region. In the dyad model, the other pair of synaptotagmin molecules would sit directly behind the pair shown here.

Figure 5. Proposed movements of the apical synaptotagmins that lead to membrane fusion. A. "Top" view of the pairs of Dsyt molecules at the vesicle-plasma membrane interface. To achieve this view, the plasma membrane has been removed and one is looking at the apical surface of a docked, "release-ready" synaptic vesicle. In this plane, only the transmembrane domains (TM) of the four synaptotagmins (red circles) and the β-strand motif (curved red line labeled linker) are evident. B. Lateral movement of the apical synaptotagmin dimers: This image shows an early phase of the predicted lateral separation of the membrane-spanning domains of

the synaptotagmin pairs that is presumed to drive membrane fusion. This lateral movement is postulated to occur as part of a multi-step process: first, Ca^{2+} binds to the C2 domains of the synaptotagmins; second, this leads to a translocation of the Ca^{2+}-liganded C2 domains from the cytosol and their insertion into the plasma membrane; third, this movement of the C2 domains is transmitted through the polypeptide backbone of each synaptotagmin molecule and causes the lateral separation and rotational pivoting of the TM domains. In this schematic, the TM domains are shown before they start to pivot. C. Pivoting of the TM domains: The lateral movement of the apical synaptotagmins (shown in C) causes the TM domains of each of the four synaptotagmins to pivot 90^o with respect to the plane of the vesicle membrane. The TM domains will transiently reach the state shown here before continuing to pivot until each TM domain reaches an orientation that is 180^o relative to its original resting state (in A). See Gundersen and Umbach (2013 for details).

Figure 6. Variation of the dyad scheme in which the synaptotagmins act as monomers rather than dimers. In this alternative scenario, each of the apical synaptotagmins (same view as in Fig.5) acts autonomously. Thus, starting from the same position as shown in Fig. 5A (with the caveat that the exact angle at which the polypeptide backbone of each synaptotagmin molecule projects radially from the TM domains is not specified), Ca^{2+} entry into the nerve terminal will induce the synaptotagmins to transition to the point shown in A (for the same reasons given in Fig.5). Although the TM domains of each of these synaptotagmins also should begin to pivot, the view shown here is given in order to emphasize the radial separation of the synaptotagmin monomers. Then, in B, the TM domains are shown having pivoted by 90^o for the same reason as shown in Fig.5C.

Figure 1

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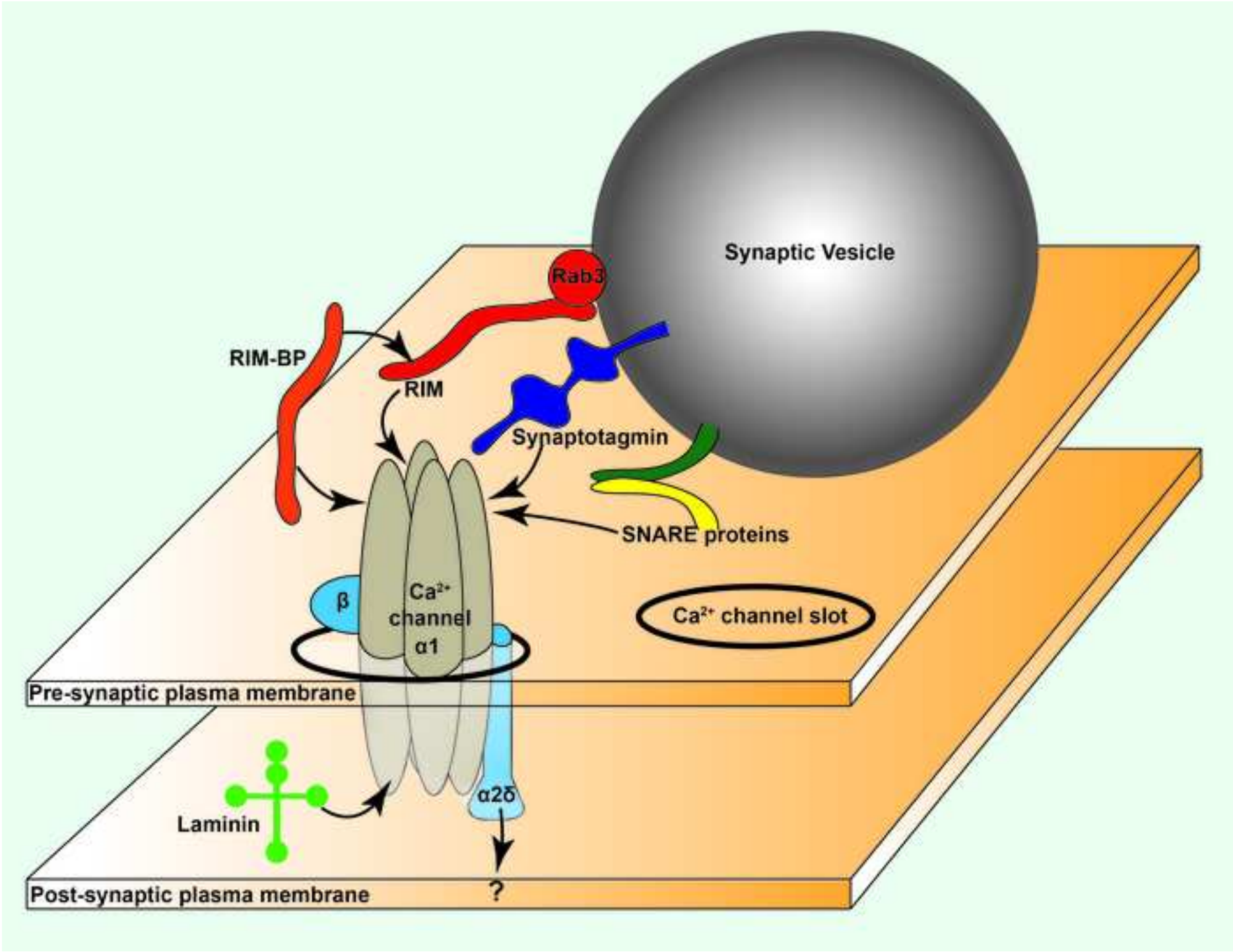

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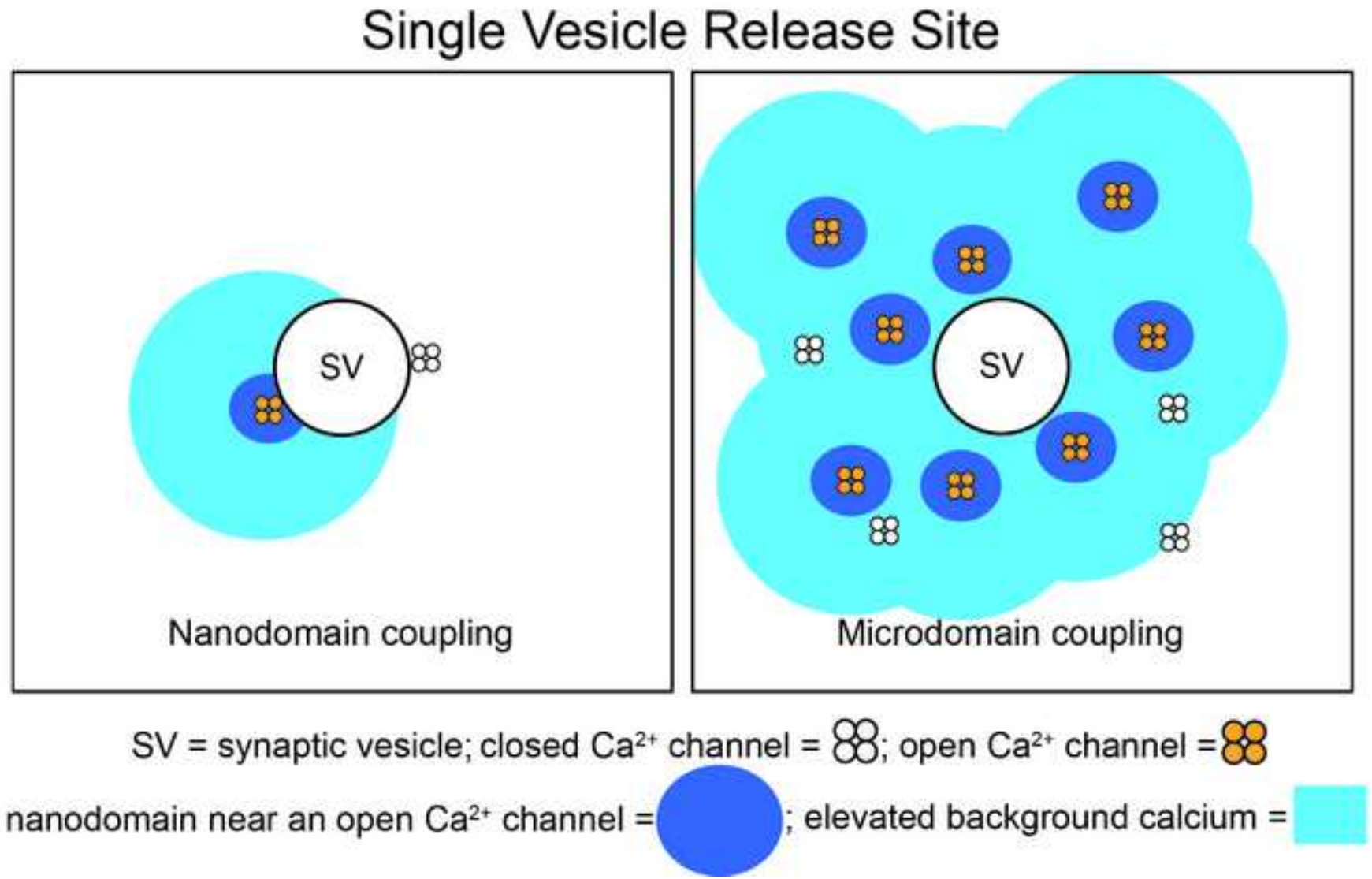

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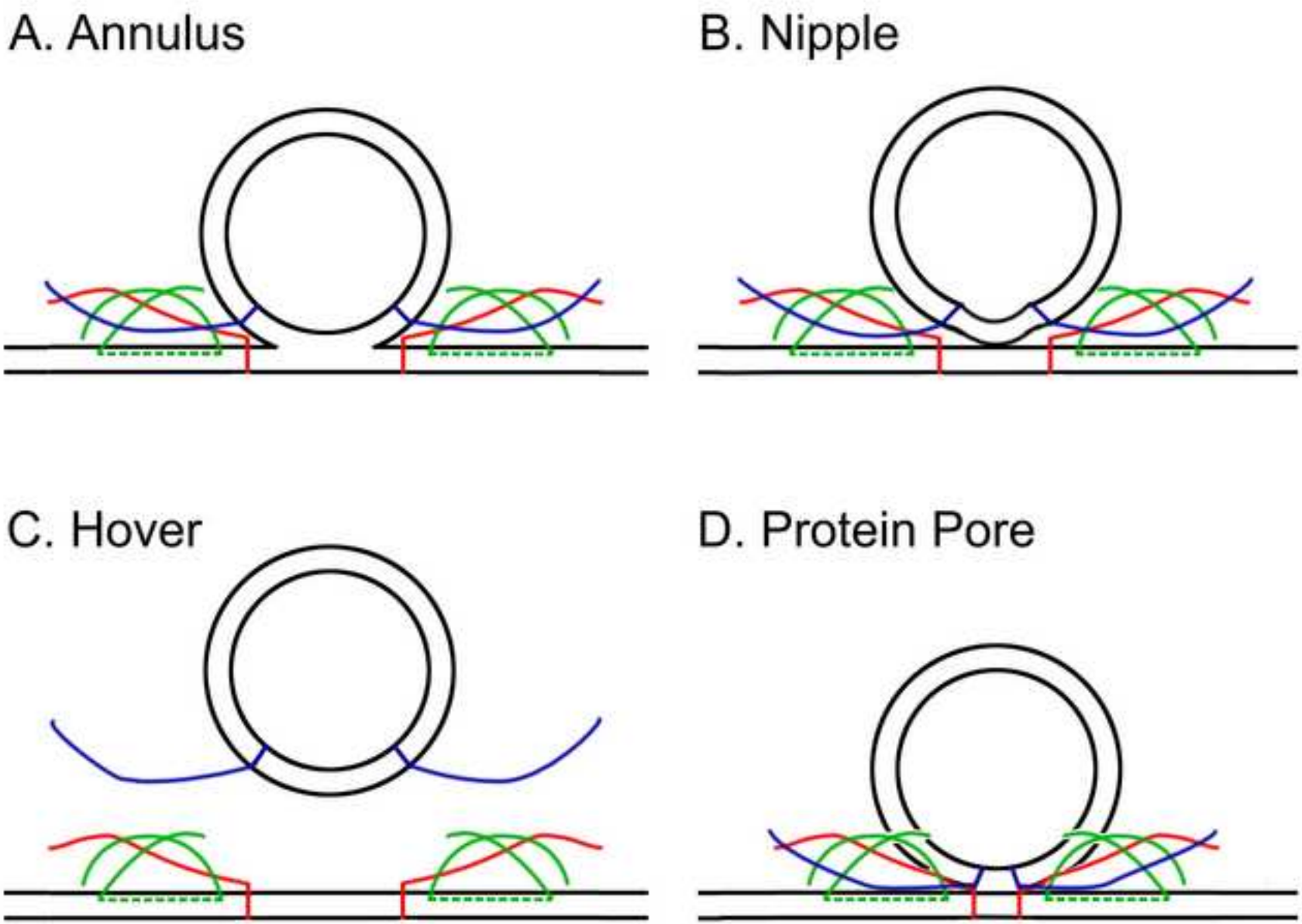

Figure 4

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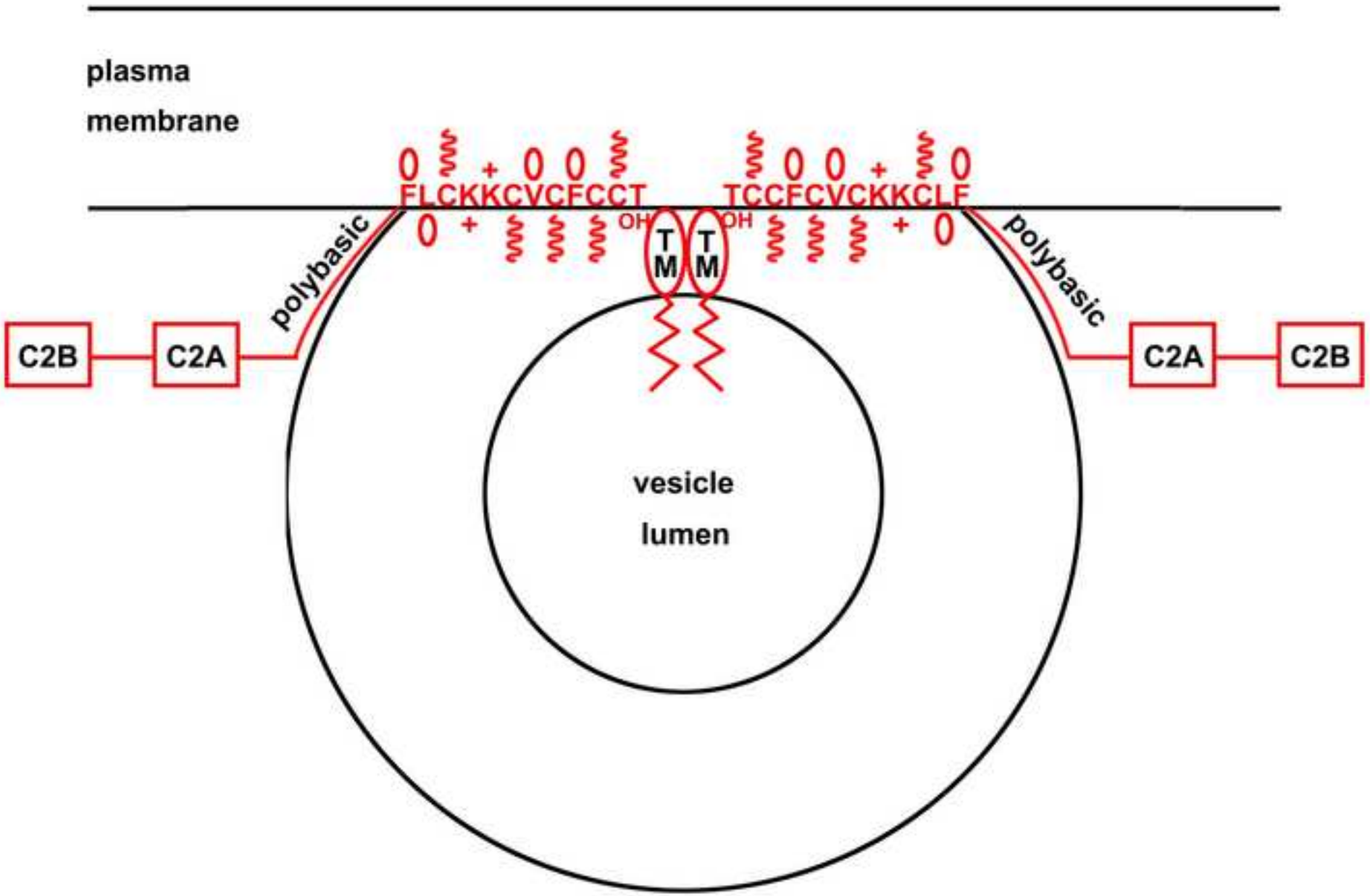

Figure 5

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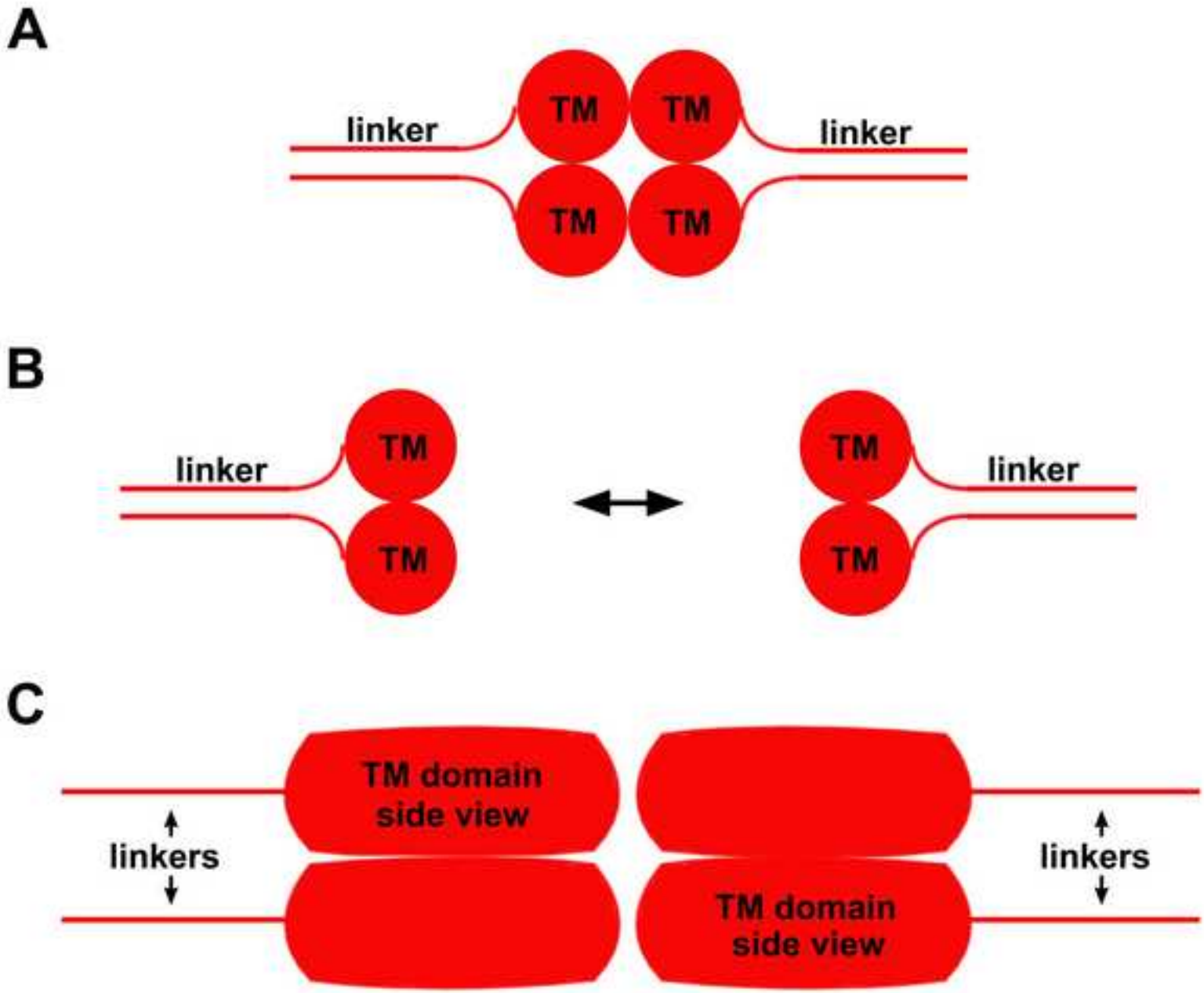

Figure 6

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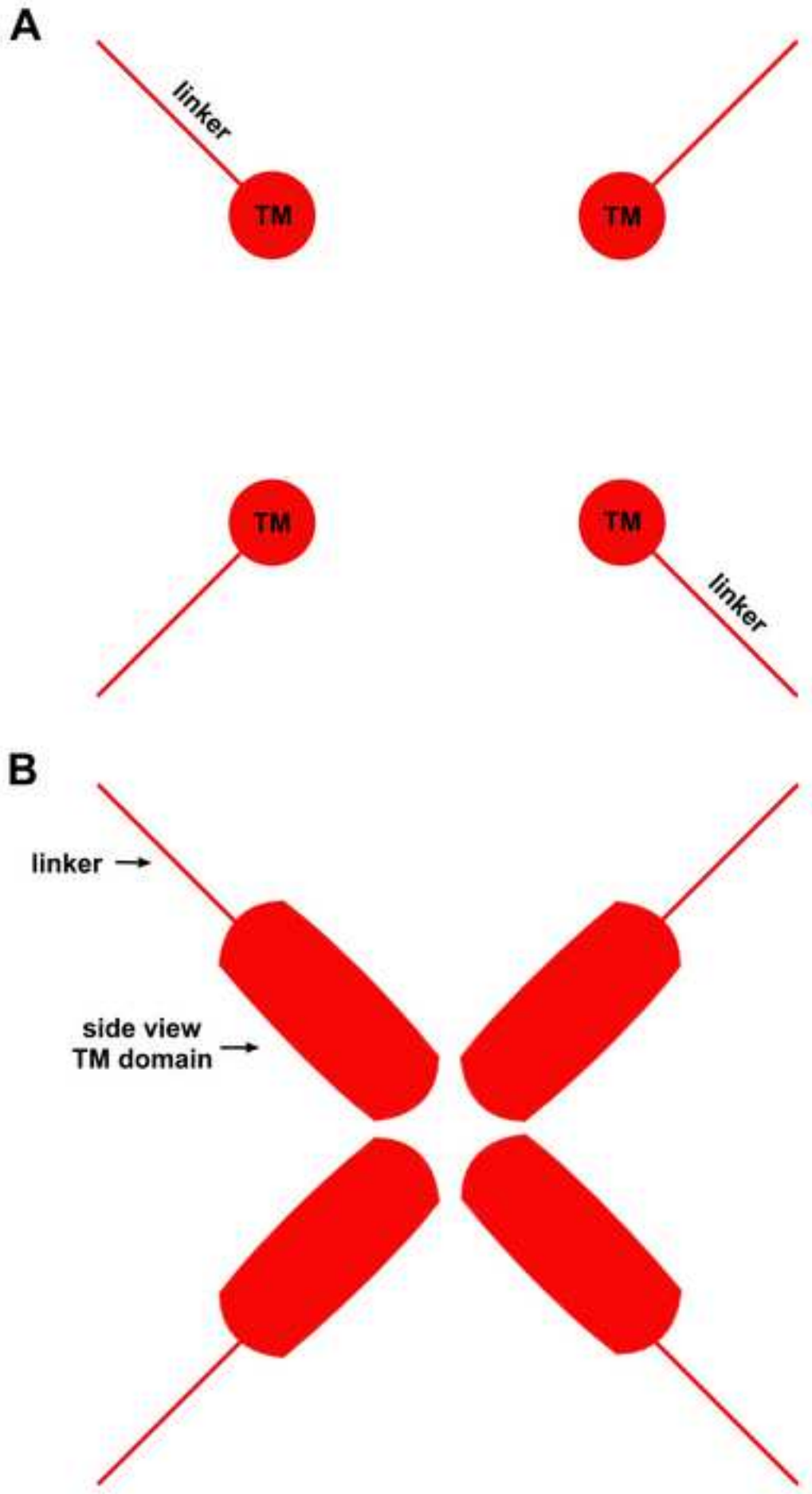

***Abbreviation List**

Abbreviations: Ca^{2+}, calcium; Rab, ras like in rat brain; RIM, Rab3 interacting molecule; RIM-BP, Rab3 interacting molecule binding protein; CAST, Cytomatrix at the active zone-associated structural protein; MIDAS, metal ion-dependent adhesion site; SNARE, soluble, N-ethylmaleimide-sensitive factor attachment receptor; VAMP, vesicle associated membrane protein; SNAP-25, synaptosome-associated protein of 25 kDa; TIRF, total internal reflection fluorescence; GFP, green fluorescent protein; PKC, protein kinase C; PIP2, phosphatidylinositol bisphosphate; KO, knockout; WT, wild-type.