Is synaptotagmin the calcium sensor?

Motojiro Yoshihara, Bill Adolfsen and J Troy Littleton*

After much debate, recent progress indicates that the synaptic vesicle protein synaptotagmin I probably functions as the calcium sensor for synchronous neurotransmitter release. Following calcium influx into presynaptic terminals, synaptotagmin I rapidly triggers the fusion of synaptic vesicles with the plasma membrane and underlies the fourth-order calcium cooperativity of release. Biochemical and genetic studies suggest that lipid and SNARE interactions underlie synaptotagmin's ability to mediate the incredible speed of vesicle fusion that is the hallmark of fast synaptic transmission.

Addresses
Picower Center for Learning and Memory, Department of Biology and Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
*e-mail: troy@mit.edu

Current Opinion in Neurobiology 2003, **13**:315–323

This review comes from a themed issue on
Signalling mechanisms
Edited by Morgan Sheng and Terrance P Snutch

0959-4388/03/$ – see front matter

DOI 10.1016/S0959-4388(03)00063-1

Introduction

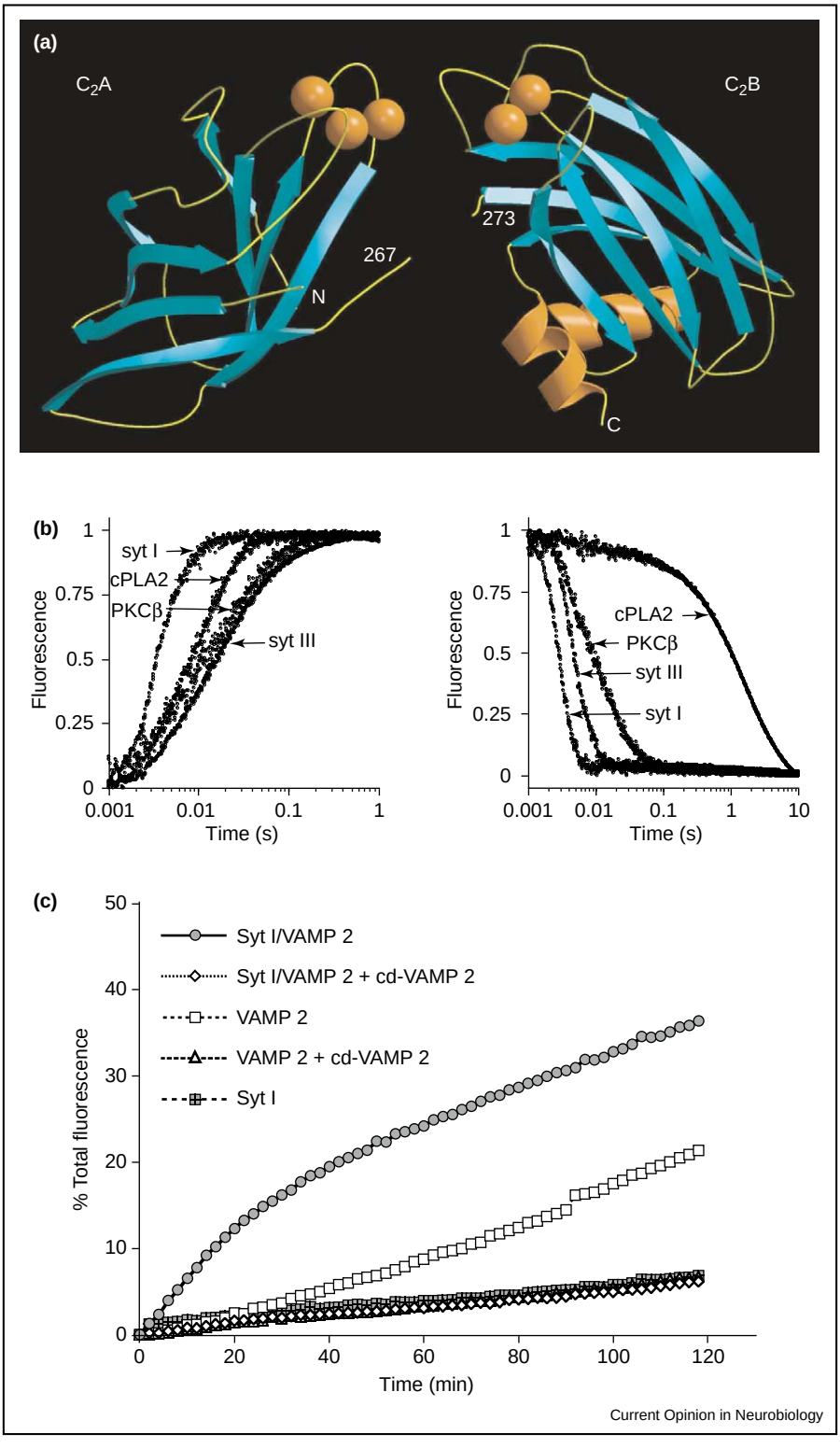
Intercellular communication at neuronal synapses occurs on a millisecond timescale and relies on the rapid calcium-triggered fusion of neurotransmitter-filled synaptic vesicles at specialized active zones in the presynaptic neuron. Following the establishment of the calcium hypotheses for neurotransmitter release by Katz and Miledi [1], multiple approaches have been undertaken to characterize the molecular mechanisms that link calcium influx to membrane fusion. Similar to most intracellular membrane-trafficking steps, synaptic-vesicle fusion requires the assembly of the SNARE complex [2–4,5•]. The plasma-membrane-associated target membrane proteins (t-SNAREs) syntaxin and SNAP-25, together with the synaptic vesicle membrane protein (v-SNARE) synaptobrevin, assemble into a four-helix bundle [6] that can bridge membranes and mediate fusion in reconstitution experiments [7]. However, fusion via reconstituted SNARE proteins is slow and calcium-independent, in contrast with synaptic transmission, where SNARE assembly and subsequent fusion is rapid and calcium-triggered [4,5•].

The search for synaptic calcium sensors that regulate SNARE-dependent fusion has largely focused on the synaptotagmins, which are transmembrane proteins containing tandem calcium-binding C2 domains (C2A and C2B) (Figure 1a). Synaptotagmin I is an abundant calcium-binding synaptic vesicle protein [8,9] that has been demonstrated via genetic studies to be important for efficient synaptic transmission *in vivo* [10–13]. The C2 domains of synaptotagmin I bind negatively-charged phospholipids in a calcium-dependent manner [9,14,15,16•–18•]. There is compelling evidence that phospholipid binding is an effector interaction in vesicle fusion, as the calcium dependence of this process ($\approx$ 74 μM) and its rapid kinetics (on a millisecond scale) (Figure 1b) fit reasonably well with the predicted requirements of synaptic transmission [15]. In addition to phospholipid binding, the calcium-stimulated interaction between synaptotagmin and the t-SNAREs syntaxin and SNAP-25 [15,19–23] provides a direct link between calcium and the fusion complex. Indeed, synaptotagmin I can accelerate SNARE-mediated liposome fusion in reconstitution experiments (Figure 1c; [24•]). The calcium-dependent oligomerization of synaptotagmin [25–28,29•] has also been hypothesized to organize SNARE complexes into a fusion pore. For additional details on the biochemical characterization of synaptotagmin, we refer the reader to several recent in-depth reviews on the topic [30–32]. Here we summarize recent studies designed to test correlations between synaptotagmin I's biochemical activities observed *in vitro* and its physiological function as measured *in vivo*.

Analysis of synaptotagmin null mutants

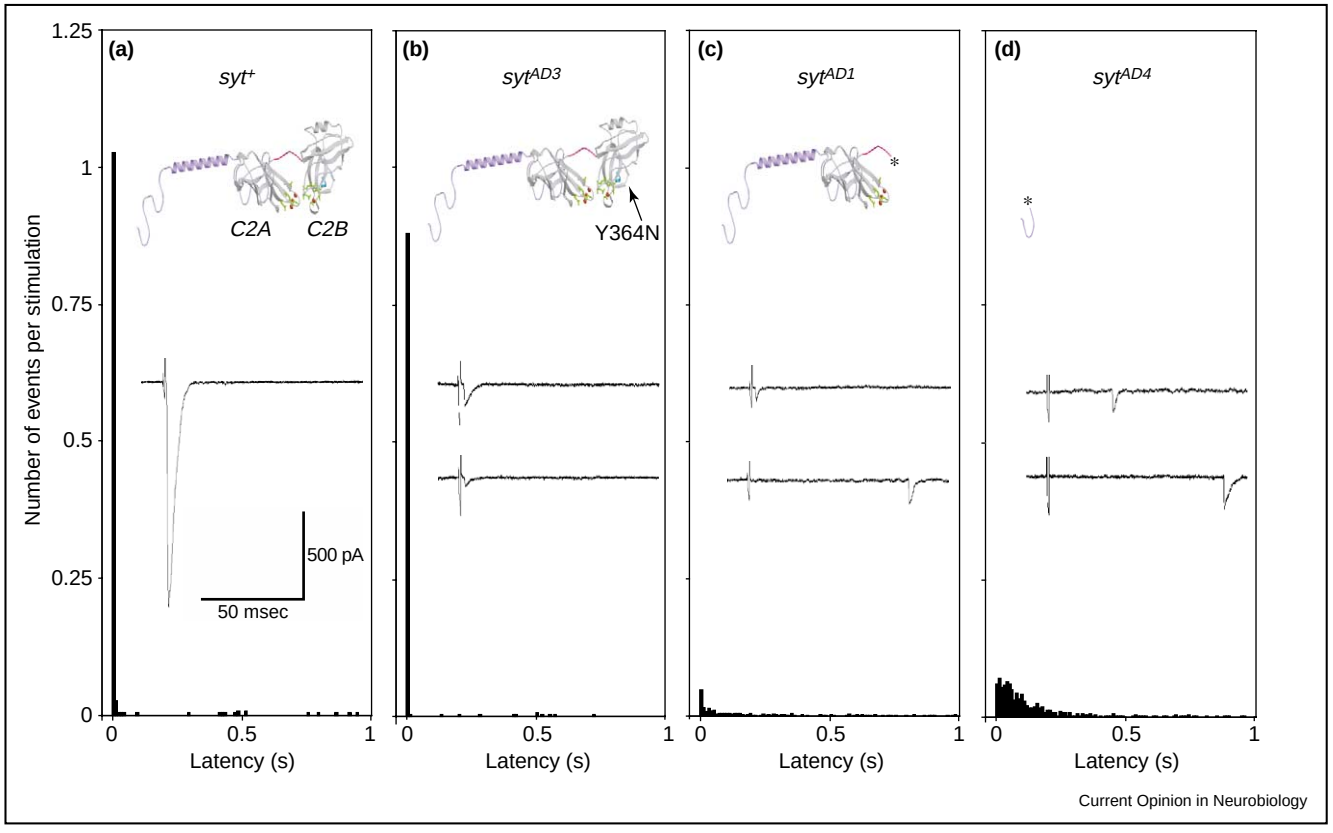
Genetic studies on the function of synaptotagmin I have established striking similarities between the loss-of-function synaptic phenotypes in mammals and invertebrates. Elimination of synaptotagmin I in mice specifically disrupts the fast synchronous phase of synaptic vesicle fusion, without altering spontaneous or latrotoxin-induced fusion [12]. Similarly, *Drosophila synaptotagmin I* null mutants (syt^{AD4}) specifically lack the synchronous component of evoked vesicle fusion, but have normal spontaneous fusion (Figure 2; [33•]). Following nerve stimulation in syt^{AD4} mutants, a kinetically distinct asynchronous component of vesicle fusion is uncovered in the complete absence of the fast synchronous phase of release. The time constant for delayed release in *synaptotagmin* null mutants is 115 ms, in sharp contrast to the time constant of a few milliseconds for synchronous release at wild type synapses. These two distinct phases of transmitter release — a fast component completed in a few milliseconds and a slow component with a time constant of $\approx$100 ms — have also been observed in primary hippocampal neuronal cultures [34].

Figure 1



Structural and biochemical properties of synaptotagmin I. **(a)** Ribbon diagrams of the C2A and C2B domains of synaptotagmin I reveal an eight-stranded β-sandwich motif with calcium-binding loops protruding from the top surface. Reprinted from [17•] with permission from Elsevier Science. **(b)** The lipid-binding kinetics of the C2A domain of synaptotagmin I occur on a millisecond time scale compared to other slower C2 domains. Association (left panel) and disassembly (right panel) kinetics of fluorescently labeled synaptotagmin I (syt I), synaptotagmin III (syt III), PKCβ and $cPLA_2$ in the presence of labeled liposomes are shown. Reprinted from [15] with permission from Elsevier Science. **(c)** Synaptotagmin can bind

Figure 2



Latency analysis of evoked neurotransmitter release in synaptotagmin mutants. Genotypes are **(a)** wild type syt^{+}; **(b)** syt^{AD3}; **(c)** syt^{AD1}; **(d)** syt^{AD4}. The latencies of synaptic currents in 4 mM calcium for 1 s following nerve stimulation are plotted (100 stimuli for each cell). Results from each animal were averaged and presented as the number of events occurring in 10 millisecond intervals following stimulation. A predicted model for synaptotagmin's structure in each of the three mutants is indicated above each plot. In the absence of synaptotagmin I, synchronous release is abolished and a kinetically distinct delayed asynchronous release pathway is uncovered. Reprinted from [33•] with permission from Elsevier Science.

It has been demonstrated that vesicle release requires four or more calcium ions to trigger fusion of one synaptic vesicle (fourth-order cooperativity). Both of these kinetic phases of release have a steep fourth-order calcium cooperativity [34,35], suggesting that two independent sensors with similar calcium cooperativities, yet unique calcium affinities, coexist at synapses. The intact cooperativity of residual release previously reported in *synaptotagmin* null mutants [36] was one of the major arguments against synaptotagmin functioning as a calcium sensor. However, this study did not differentiate between the cooperativity of fast release versus that of asynchronous release. Previous measurements reflected the cooperativity of the asynchronous mechanism [36], as no synchronous component remains in the null mutant [33•], and cooperativity for synchronous fusion cannot be measured. The two-calcium-sensor model is also applicable to the fusion of large dense-core vesicles in chromaffin cells, where removal of synaptotagmin selectively eliminates the fast fusion of readily releasable vesicles during the exocytotic burst without affecting the fusion of the slowly releasable pool [37•]. Whether the slowly releasable phase of the fusion of large dense-core vesicles corresponds to the asynchronous fusion of small synaptic vesicles is unknown. Together, these studies suggest that two calcium sensors with distinct calcium sensitivities are capable of triggering fusion and that synaptotagmin I encodes or is required for the function of the fast calcium sensor.

(Figure 1 Legend Continued) t-SNAREs at all stages of SNARE assembly and accelerates fusion between liposomes containing reconstituted SNAREs (gray circles). Controls include SNARE-mediated fusion in the absence of synaptotagmin I (empty squares), reactions containing a dominant-negative cytoplasmic domain of VAMP 2/synaptobrevin 2 (cd-VAMP 2; clear diamonds and triangles) and fusion in the presence of synaptotagmin only (no VAMP 2; gray squares). To date, these stimulatory effects have not been found to require calcium, although the establishment of millisecond timescale fusion assays may be required to observe rapid kinetic differences. Reprinted from [24•] by copyright permission from The Rockefeller University Press.

Genetic characterization of C2A function

If synaptotagmin functions as the fast calcium sensor, one would expect that disruption of the calcium-binding properties of synaptotagmin would mimic the null phenotype. Recent reports in mice [38•] and *Drosophila* [39•,40•] describe the effects of mutating C2A or C2B calcium ligands. To date, no study has examined the consequences of disrupting calcium binding to both domains simultaneously. The general approach employed in these studies was to mutate the key aspartate residues that make up the calcium-binding pocket at the exposed loops of each domain (Figure 1a). Although this direct approach seems to be the most straightforward way of determining the function of calcium binding to synaptotagmin, the phospholipid-binding properties of the C2A and C2B domain turn out to be largely redundant.

When calcium-binding residues in C2A are mutated, the calcium-dependent interactions of the isolated C2A domain are abolished. However, when the mutated C2A domain is placed in tandem with a wild-type C2B domain, the calcium-dependent interactions of the hybrid C2A–C2B protein are unaffected [16•]. This redundancy is attributable to calcium-dependent lipid binding by the C2B domain, which 'drags' the attached C2A domain into membranes and activates its lipid-binding properties [16•]. This biochemical redundancy was borne out by genetic manipulations of the C2A calcium-binding ligands. Mutations of two calcium-binding residues in the C2A domain (D232N and D238N) were generated in mice, which were then tested for biochemical and electrophysiological defects [38•]. The mutant mice have few or no electrophysiological defects and the native protein isolated from mutant animals displays normal calcium-dependent syntaxin and phospholipid interactions. Similar results were reported in a *Drosophila* mutant with a D229N substitution in C2A [39•], although quantitative correlation between release probability and synaptotagmin function with this approach is difficult because of variability in protein levels following heat-shock induction of the transgene. In summary, genetic disruptions of C2A calcium-binding ligands have relatively mild effects on release and on the calcium-dependent interactions of the full-length protein.

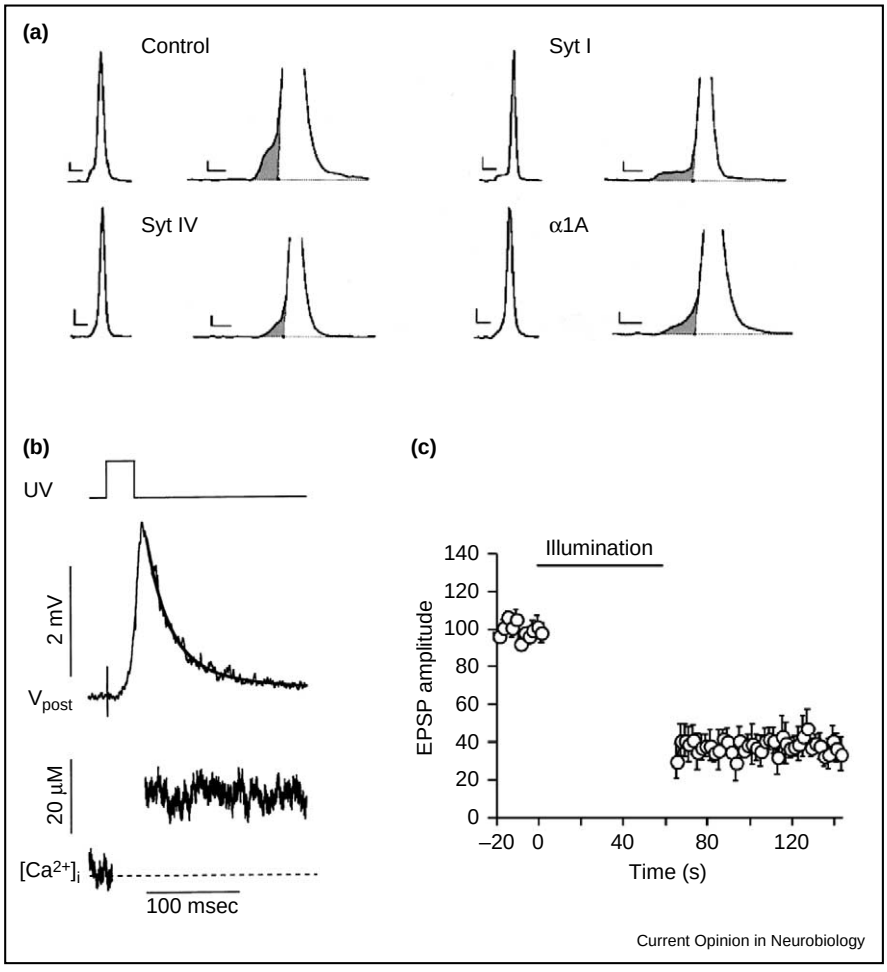
To circumvent the redundancy between the C2 domains, Fernàndez-Chacòn *et al.* [41•] engineered mutations in C2A outside the calcium binding sites in a neighboring charged arginine residue (R233Q). In the R233Q knock-in, a twofold decrease in calcium-dependent phospholipid binding by the native C2A-C2B protein was observed. Mutant mice displayed a twofold decrease in release probability at hippocampal synapses, but had no defect in the size of the readily releasable pool or in presynaptic calcium influx. A complementary approach examined the effects of a mutation (syt^{AD1}) that deletes the C2B domain in *Drosophila*, leaving only the C2A domain intact at synapses [33•]. In syt^{AD1} animals, both the synchronous and the asynchronous phases of release were present and could be kinetically separated (Figure 2), suggesting that calcium-dependent lipid binding by the C2A domain alone can trigger the fast phase of release with low release probability but cannot fully suppress slow asynchronous release. The coexistence of both release phases in this mutant also suggests that synaptotagmin I does not convert the slow asynchronous pathway into a fast synchronous one, but instead mediates the fast component of release, whereas the asynchronous pathway is mediated by a second calcium sensor. Importantly, although the C2A domain alone can trigger synchronous release, the calcium cooperativity is reduced from four to one [33•]. Reductions in the calcium cooperativity of release have also been observed in *Drosophila* larvae where synaptotagmin I has been acutely disrupted with a novel transgenic photoinactivation technique [42•], and in heteroallelic combinations of *Drosophila synaptotagmin* mutations with the syt^{AD1} allele [43].

Biochemical studies indicate that the loss of the C2B domain greatly diminishes high-affinity interactions between synaptotagmin and SNAREs [15,23]. One interpretation of this data is that the calcium cooperativity of neurotransmitter release is mediated through synaptotagmin–SNARE interactions, whereas phospholipid binding triggers rapid fusion with increased release probability. Supporting this model are recent findings indicating that synaptotagmin–SNARE interactions are essential for the membrane fusion of large dense-core vesicles in PC12 cells as well [16•,44•].

Genetic disruption of C2B functions

Given the redundancy of C2A calcium binding conferred by C2B, are any phenotypes associated with specific disruptions of the calcium-binding properties of the C2B domain? Mackler *et al.* [40•] have addressed this issue in *Drosophila* by examining transgenic strains harboring mutations in key calcium-binding residues (D416N, D418N and a D356N, D362N double mutant) within the C2B domain. In contrast to disruptions of C2A calcium binding, mutations in C2B result in dramatic defects in vesicle fusion. However, the phenotypes are even more severe than those seen in the null mutant or in animals completely lacking the C2B domain, indicating the transgenes function as dominant-negative inhibitors of vesicle fusion [40•]. Indeed, expression of the D356N, D362N transgene in wild-type animals containing native synaptotagmin resulted in >90% inhibition of release. Although it is difficult to extrapolate loss-of-function phenotypes secondary to the dominant-negative effects, electron microscopy performed on the C2B mutants indicate an abundance of docked vesicles, supporting the argument that the dominant-negative C2B transgenes block release following vesicle docking. Similar results using acute inactivation of synaptotagmin also support a

Figure 3



Regulation of vesicle fusion by synaptotagmin I. **(a)** Synaptotagmin I stabilizes fusion pore opening during large dense-core vesicle exocytosis, leading to longer prespike foot currents. The different scales illustrate the whole spike (left) and the foot current (right and shaded). Scale bars: 10 pA, 2 ms, left; 5 pA, 1 ms, right. Reprinted with permission from [47•] (copyright [2001] American Association for the Advancement of Science). **(b)** The photolysis of caged calcium (top panel) at the squid giant synapse results in a decay of the excitatory postsynaptic potential (middle panel) even though a sustained elevation of calcium remains (bottom panel). Reprinted from [49] with permission from Elsevier Science. **(c)** Using photoinactivation to acutely disrupt transgenic synaptotagmin I at *Drosophila* synapses leads to a rapid block of exocytosis. Photoinactivation was performed in the absence of stimulation. Following resumption of stimulation, release is decreased by the maximum amount at the first stimulus and there is no stimulation-dependent decay or temporal requirement for the defect to manifest. These findings strongly suggest that synaptotagmin I functions at the fusion step and does not resupply the vesicle pool or function to maintain docked vesicles. Reprinted from [42•] with permission from Elsevier Science.

post-docking function for synaptotagmin (Figure 3c) [42•]. The C2B dominant-negative transgenes also suppress the asynchronous release observed in the null mutant, without a corresponding increase in synchronous fusion. These findings suggest that the function of synaptotagmin I in suppressing asynchronous release is distinct and genetically separable from its role in triggering fast synchronous fusion.

To circumvent the dominant-negative effects, a mutated tyrosine residue in the C2B domain near the calcium-binding ligands has been characterized (syt^{AD3}) that eliminates conformational changes induced by calcium binding to C2B but does not have dominant-negative effects [29•,33•]. This point mutation (Y364N) disrupts the calcium-dependent oligomerization of synaptotagmin mediated through its C2B domain [29•,45] but does not abolish C2A phospholipid binding or SNARE interactions. Animals containing the Y364N mutant have a 10-fold reduction in synchronous fusion but normal calcium cooperativity. The release that is still observed in the Y364N mutant is completely synchronous with nerve stimulation and does not have an asynchronous component (Figure 2), similar to what is observed in the wild type. These findings suggest that calcium-dependent oligomerization via the C2B domain enhances the sensitivity

of vesicle fusion to calcium, but is not required for cooperativity or the suppression of the slow asynchronous phase of release.

Suppression of asynchronous release by synaptotagmin

The delayed asynchronous release pathway is rarely observed in wild type *Drosophila* but is fully uncovered in *synaptotagmin* null mutants [33•]. Although the fast and slow components of neurotransmitter release are mediated by distinct mechanisms and calcium sensors, synaptotagmin I normally suppresses the asynchronous component of neurotransmitter release (Figure 2). Zucker and colleagues [46] have postulated two separate calcium sensors in order to explain the short-term facilitation induced by residual calcium. Genetic evidence indicates that synaptotagmin I is the low-affinity calcium sensor for synchronous release. A second high-affinity sensor with slower kinetics is postulated to be responsible for both facilitation and the asynchronous phase of release [46]. Convincing evidence that separate molecular pathways underlie the two release phases is provided by the observation that *Drosophila synaptotagmin* mutants that lack the C2B domain (syt^{AD1}) show both phases of release, whereas a C2B point mutant (syt^{AD3}) shows intact suppression of the asynchronous pathway but is still defective in triggering synchronous fusion (Figure 2). syt^{AD3} mutants also exhibit far fewer fusion events evoked by salines containing high potassium or calcium ionophores than are observed in the null mutant syt^{AD4}, where there is increased asynchronous release because of the unrestricted activity of the high-affinity calcium sensor in the absence of synaptotagmin [33•].

What potential mechanisms underlie the suppression of asynchronous release? One possibility is suggested by the recent finding that synaptotagmin I stabilizes fusion pores during dense-core vesicle release in PC12 cells [47•]. Wang *et al.* observed that overexpression of synaptotagmin I in PC12 cells increased the duration of the small foot current that precedes the major release spike measured in amperometric recordings (Figure 3a). The foot current is postulated to arise from the release of a small amount of transmitter during the initial assembly of the fusion pore before it expands completely during full fusion. The increased foot duration suggests synaptotagmin I can stabilize the fusion complex and decrease the free energy needed for dilation of the fusion pore [48]. A similar stabilization of the fusion complex of synaptic vesicles could account for the suppression of asynchronous release by synaptotagmin. By analogy with the inactivation of voltage-gated channels and the desensitization of ligand-gated channels, the initial influx of calcium may trigger synaptotagmin/SNARE-dependent conformational changes that enhance membrane fusion for several milliseconds. A secondary phase of inhibitory conformational changes in synaptotagmin may then occur several milliseconds later, resulting in fusion pores that have a lower probability of dilating. This would effectively serve to drive rapid synchronous fusion while inhibiting the asynchronous release normally activated by the high-affinity calcium sensor. Electrophysiological studies using the squid giant synapse have demonstrated that release rates adapt and decay within tens of milliseconds after the initial calcium influx, even during sustained elevated intracellular calcium concentrations obtained using caged calcium (Figure 3b; [49]). These results cannot be explained by vesicle depletion, suggesting that an intrinsic property of the fusion machinery is likely to change following the initial calcium spike. It is tempting, on the basis of the ability of C2B calcium ligand mutants to dominantly suppress asynchronous release, to speculate that the C2B domain may be the site of such secondary inhibitory conformational changes [40•]. The suppression of the asynchronous release pathway, together with the steep calcium cooperativity provided by synaptotagmin I, probably explains the sharp time resolution of nerve-evoked release.

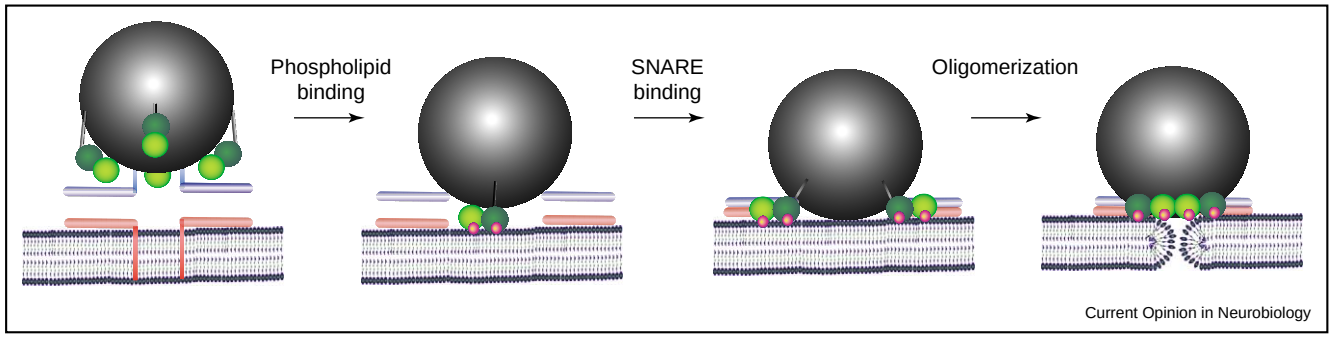
Conclusions

Katz hypothesized the existence of the 'calcium receptor' three decades ago in his classical description of the calcium hypothesis of neurotransmission. The explosion of molecular cloning and reverse genetics in the 1990s provided a host of candidate molecules that are enriched at presynaptic terminals and important for neurotransmitter release. After debate about the roles of many of these molecules, we have finally reached a point where most data suggest that synaptotagmin I is the fast calcium sensor for synchronous neurotransmitter release. The past several years have seen several effector interactions, including SNARE binding, phospholipid binding and oligomerization, emerge as potential underlying mechanisms. Over the next few years there is likely to be enthusiastic debate about which of these interactions are relevant *in vivo*. As a working hypothesis to be tested over the next few years, we propose a three-stage model for calcium-activated vesicle fusion mediated by synaptotagmin as shown in Figure 4. We expect that this model will be polished by experimental results using systematic structure–function studies.

Update

Several recent findings have provided insights into the role of synaptotagmin's effector interactions during vesicle fusion. Rickman and Davletov [50•] demonstrate that native synaptotagmin I is capable of binding a t-SNARE heterodimer of syntaxin and SNAP-25, even in the absence of calcium. The interaction requires the presence of both C2 domains of synaptotagmin, suggesting that tandem C2 domains may have unique functions not observed in isolated C2 domains. Ernst and Brunger [51] have recently solved the crystal structure of a truncated SNARE complex and demonstrate that synaptotagmin recombinant

Figure 4



A three-stage model for calcium-dependent synaptic vesicle fusion mediated by synaptotagmin I. In the complete absence of synaptotagmin, no synchronous release is observed. Calcium-dependent phospholipid binding (calcium is represented by magenta circles) mediated by the C2 domains can trigger synchronous vesicle fusion. In the absence of high-affinity interactions with the SNARE complex, synaptotagmin's calcium-dependent phospholipid binding ability alone has a low release probability. The calcium-dependent binding of synaptotagmin to SNAREs may underlie the calcium cooperativity of release and serve to increase release probability. Cooperativity could be accounted for by multiple calcium ions binding to one synaptotagmin monomer and triggering SNARE association or by the requirement of multiple synaptotagmin–SNARE interactions per fusion event. Wild-type levels of release would require calcium binding to the C2B domain and subsequent oligomerization to maximize release probability. Oligomerization may serve to focus multiple synaptotagmin–SNARE complexes at the interface of the merging lipid bilayers. Although the model is drawn stepwise for clarity, the actual sequence of molecular interactions *in vivo* is unknown. Synaptotagmin would also function to suppress the asynchronous phase of release. Calcium-independent interactions between synaptotagmin and t-SNAREs may also function in vesicle fusion, but their precise role in the pathway is still unknown. The C2A domain of synaptotagmin is represented by the dark green circle and the C2B domain is shown in light green. The v-SNARE synaptobrevin is shown in blue and the t-SNARE syntaxin is shown in red. Modified from [33•] with permission from Elsevier Science.

proteins containing both C2 domains interact with the truncated SNARE complex, even in the absence of calcium. Together with previous data, these findings suggest that synaptotagmin-SNARE interactions may have both calcium-dependent and calcium-independent roles in fusion. To test the *in vivo* role of synaptotagmin-SNARE interactions, Shin *et al.* [52•] have analyzed the effects of strontium on triggering release in synaptotagmin knockout mice. Although strontium is much less effective at triggering fusion compared to calcium, strontium-stimulated release is also defective in synaptotagmin knockouts. The authors demonstrate that strontium binds specifically to the C2B domain of synaptotagmin and can induce lipid binding, but does not effect SNARE interactions. The authors propose that strontium's ability to trigger fast release requires synaptotagmin–phospholipid interactions, but not SNARE binding. Further studies will be required to determine if the increased release efficacy of calcium versus strontium is due to calcium's ability to promote high affinity synaptotagmin-SNARE interactions. In addition to lipid and SNARE binding, calcium-induced oligomerization of synaptotagmin I has been suggested to be an important step in fusion. Wu *et al.* [53] provide the first images of calcium-dependent synaptotagmin oligomers assembled on phospholipid bilayers. Recombinant synaptotagmin assembles into heptameric oligomers in the presence of calcium, suggesting oligomerization might play a role in regulation of fusion pore opening or dilation. Future studies will no doubt continue to elucidate the role of these synaptotagmin effector interactions during vesicle fusion.

Acknowledgements

Work from the authors' laboratory was supported by grants from the NIH, the Human Frontiers Science Program Organization and the Searle Scholars Program. J Troy Littleton is a Alfred P Sloan Research Fellow.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Katz B, Miledi R: **The timing of calcium action during neuromuscular transmission**. *J Physiol* 1967, **189**:535-544.

2. Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE: **SNAP receptors implicated in vesicle targeting and fusion**. *Nature* 1993, **362**:318-324.

3. Littleton JT, Chapman ER, Kreber R, Garment MB, Carlson SD, Ganetzky B: **Temperature-sensitive paralytic mutations demonstrate that synaptic exocytosis requires SNARE complex assembly and disassembly**. *Neuron* 1998, **21**:401-413.

4. Chen YA, Scales SJ, Patel SM, Doung YC, Scheller RH: **SNARE complex formation is triggered by Ca^{2+} and drives membrane fusion**. *Cell* 1999, **97**:165-174.

5. • Hu K, Carroll J, Fedorovich S, Rickman C, Sukhodub A, Davletov B: **Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion**. *Nature* 2002, **415**:646-650.
This study employed a fluorescence-based fusion assay to study the interactions between synaptobrevin from purified synaptic vesicles and t-SNAREs reconstituted into liposomes. Calcium rapidly triggers both the formation of SNARE complexes and vesicular fusion in this assay. However, soluble syntaxin and SNAP-25 fail to form SNARE complexes when incubated with synaptobrevin present on synaptic vesicles, suggesting that synaptobrevin is normally restricted from interacting with its SNARE partners. The authors present a model in which calcium binding by synaptotagmin leads to close membrane opposition, thereby bringing t-SNAREs into close contact with synaptobrevin and triggering SNARE complex formation and fusion.

6. Sutton RB, Fasshauer D, Jahn R, Brunger AT: **Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution**. *Nature* 1998, **395**:347-353.

7. Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Söllner TH, Rothman JE: **SNAREpins: minimal machinery for membrane fusion**. *Cell* 1998, **92**:759-772.

8. Perin MS, Fried VA, Mignery GA, Jahn R, Südhof TC: **Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C**. *Nature* 1990, **345**:260-263.

9. Brose N, Petrenko AG, Südhof TC, Jahn R: **Synaptotagmin: a calcium sensor on the synaptic vesicle surface**. *Science* 1992, **256**:1021-1025.

10. Littleton JT, Stern M, Schulze K, Perin M, Bellen HJ: **Mutational analysis of *Drosophila* synaptotagmin demonstrates its essential role in Ca^{2+}-activated neurotransmitter release**. *Cell* 1993, **74**:1125-1134.

11. Nonet ML, Grundahl K, Meyer BJ, Rand JB: **Synaptic function is impaired but not eliminated in *C. elegans* mutants lacking synaptotagmin**. *Cell* 1993, **73**:1291-1305.

12. Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Südhof TC: **Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse**. *Cell* 1994, **79**:717-727.

13. DiAntonio A, Schwarz TL: **The effect on synaptic physiology of synaptotagmin mutations in *Drosophila***. *Neuron* 1994, **12**:909-920.

14. Davletov BA, Südhof TC: **A single C2 domain from synaptotagmin I is sufficient for high affinity Ca^{2+}/phospholipid binding**. *J Biol Chem* 1993, **268**:26386-26390.

15. Davis AF, Bai J, Fasshauer D, Wolowick MJ, Lewis JL, Chapman ER: **Kinetics of synaptotagmin responses to Ca^{2+} and assembly with the core SNARE complex onto membranes**. *Neuron* 1999, **24**:363-376.

16. • Earles CA, Bai J, Wang P, Chapman ER: **The tandem C2 domains of synaptotagmin contain redundant Ca^{2+} binding sites that cooperate to engage t-SNAREs and trigger exocytosis**. *J Cell Biol* 2001, **154**:1117-1123.

This study uses real-time voltammetry measurements from cracked PC12 cells to demonstrate that exogenously added C2A–C2B or C2A–C2A domains displace native synaptotagmin from SNARE complexes and rapidly inhibit fusion. The authors also identify redundancy in the calcium-dependent interaction with SNAREs and phospholipids by the two C2 domains, such that calcium ligand mutations in one C2 domain can be partially alleviated by the presence of a normal adjacent C2 domain.

17. • Fernandez I, Arac D, Ubach J, Gerber SH, Shin O, Gao Y, Anderson RG, Südhof TC, Rizo J: **Three-dimensional structure of the synaptotagmin 1 C2B-domain: synaptotagmin 1 as a phospholipid binding machine**. *Neuron* 2001, **32**:1057-1069.

In this study, the authors use NMR to solve the structure of the C2B domain of synaptotagmin I, demonstrating a β sandwich motif with both similarities and differences to the previously characterized C2A domain. The authors show that the C2B domain also displays calcium-dependent phospholipid interactions similar to C2A.

18. • Bai J, Wang P, Chapman ER: **C2A activates a cryptic Ca^{2+}-triggered membrane penetration activity within the C2B domain of synaptotagmin I**. *Proc Natl Acad Sci USA* 2002, **99**:1665-1670.

This study uses fluorescent reporters introduced into the calcium binding loops of C2A and C2B to reveal that both domains penetrate lipid bilayers in a calcium-dependent fashion with rapid kinetics. The ability of the C2B domain to penetrate lipid bilayers is dependent on the presence, but not the calcium-binding activity, of the adjacent C2A domain, suggesting novel cooperative interactions between tandem C2 domain proteins.

19. Bennett MK, Calakos N, Scheller RH: **Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones**. *Science* 1992, **257**:255-259.

20. Chapman ER, Hanson PI, An S, Jahn R: **Ca^{2+} regulates the interaction between synaptotagmin and syntaxin 1**. *J Biol Chem* 1995, **270**:23667-23671.

21. Li C, Ullrich B, Zhang JZ, Anderson RG, Brose N, Südhof TC: **Ca^{2+}-dependent and -independent activities of neural and non-neural synaptotagmins**. *Nature* 1995, **375**:594-599.

22. Schiavo G, Stenbeck G, Rothman JE, Söllner TH: **Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses**. *Proc Natl Acad Sci USA* 1997, **94**:997-1001.

23. Gerona RR, Larsen EC, Kowalchyk JA, Martin TF: **The C terminus of SNAP25 is essential for Ca^{2+}-dependent binding of synaptotagmin to SNARE complexes**. *J Biol Chem* 2000, **275**:6328-6336.

24. • Mahal LK, Sequeira SM, Gureasko JM, Söllner TH: **Calcium-independent stimulation of membrane fusion and SNAREpin formation by synaptotagmin I**. *J Cell Biol* 2002, **158**:273-282.

Using the well-characterized SNARE-mediated liposome fusion assay, the authors demonstrate that synaptotagmin I can enhance the rate of SNARE-dependent fusion of liposomes *in vitro*. The enhancement is seen when soluble synaptotagmin I is incubated with the preparation or when synaptotagmin is present on v-SNARE liposomes, suggesting synaptotagmin's interaction with SNAREs is topologically restricted. Calcium did not alter the rates of fusion in this assay, suggesting that the calcium-independent properties of synaptotagmin may also be important in promoting bilayer fusion.

25. Chapman ER, An S, Edwardson JM, Jahn R: **A novel function for the second C2 domain of synaptotagmin. Ca^{2+}-triggered dimerization**. *J Biol Chem* 1996, **271**:5844-5849.

26. Sugita S, Hata Y, Südhof TC: **Distinct Ca^{2+}-dependent properties of the first and second C2-domains of synaptotagmin I**. *J Biol Chem* 1996, **271**:1262-1265.

27. Littleton JT, Serano TL, Rubin GM, Ganetzky B, Chapman ER: **Synaptic function modulated by changes in the ratio of synaptotagmin I and IV**. *Nature* 1999, **400**:757-760.

28. Osborne SL, Herreros J, Bastiaens PI, Schiavo G: **Calcium-dependent oligomerization of synaptotagmins I and II. Synaptotagmins I and II are localized on the same synaptic vesicle and heterodimerize in the presence of calcium**. *J Biol Chem* 1999, **274**:59-66.

29. • Littleton JT, Bai J, Vyas B, Desai R, Baltus AE, Garment MB, Carlson SD, Ganetzky B, Chapman ER: ***synaptotagmin* mutants reveal essential functions for the C2B domain in Ca^{2+}-triggered fusion and recycling of synaptic vesicles *in vivo***. *J Neurosci* 2001, **21**:1421-1433.

This study demonstrates that synaptotagmin can bind t-SNAREs at all stages of SNARE assembly and accelerate their maturation into SDS-resistant SNARE complexes *in vitro*. The authors show that synaptotagmin can oligomerize and bind SNARE complexes simultaneously. In addition, a mutation in the C2B domain of *Drosophila* synaptotagmin that disrupts neurotransmitter release was shown to block calcium-triggered conformational changes in C2B and subsequent oligomerization. The authors hypothesize that oligomerization via the C2B domain of synaptotagmin may trigger synaptic vesicle fusion through the assembly and clustering of SNARE complexes.

30. Chapman ER: **Synaptotagmin: a Ca^{2+} sensor that triggers exocytosis?** *Nat Rev Mol Cell Biol* 2002, **3**:498-508.

31. Tucker WC, Chapman ER: **Role of synaptotagmin in Ca^{2+}-triggered exocytosis**. *Biochem J* 2002, **366**:1-13.

32. Rizo J, Südhof TC: **C2-domains, structure and function of a universal Ca^{2+}-binding domain**. *J Biol Chem* 1998, **273**:15879-15882.

33. • Yoshihara M, Littleton JT: **Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release**. *Neuron* 2002, **36**:897-908.

This study describes a structure–function analysis of an allelic series of synaptotagmin mutants in *Drosophila*. The authors demonstrate that the loss of synaptotagmin abolishes evoked synchronous release and uncovers a slow asynchronous fusion pathway. In addition, the authors provide the first conclusive demonstration of an alteration in the calcium cooperativity of the fast phase of neurotransmitter release in a synaptotagmin partial-loss-of-function mutant. The study indicates that two calcium sensors function in synaptic transmission and that synaptotagmin I functions as the fast calcium sensor. Interactions with lipid and SNAREs, as well as oligomerization, are postulated to underlie the ability of synaptotagmin to trigger rapid synchronous fusion.

34. Goda Y, Stevens CF: **Two components of transmitter release at a central synapse**. *Proc Natl Acad Sci USA* 1994, **91**:12942-12946.

35. Dodge FA Jr, Rahamimoff R: **Co-operative action of calcium ions in transmitter release at the neuromuscular junction**. *J Physiol* 1967, **193**:419-432.

36. Broadie K, Bellen HJ, DiAntonio A, Littleton JT, Schwarz TL: **Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission**. *Proc Natl Acad Sci USA* 1994, **91**:10727-10731.

37. • Voets T, Moser T, Lund PE, Chow RH, Geppert M, Südhof TC, Neher E: **Intracellular calcium dependence of large dense-core vesicle exocytosis in the absence of synaptotagmin I**. *Proc Natl Acad Sci USA* 2001, **98**:11680-11685.
In this study the authors measure the kinetics of release in adrenal chromaffin cells from synaptotagmin I knockout mice. Release from control cells is best fit by a double exponential, indicating a fast and slow release phase. Only the fast release phase is eliminated in the absence of synaptotagmin.

38. • Fernàndez-Chacòn R, Shin OH, Königstorfer A, Matos MF, Meyer AC, Garcia J, Gerber SH, Rizo J, Südhof TC, Rosenmund C: **Structure/function analysis of Ca^{2+} binding to the C2A domain of synaptotagmin 1**. *J Neurosci* 2002, **22**:8438-8446.
This study uses knock-in mice to examine synaptic function in transgenics where calcium binding to the C2A domain of synaptotagmin I is disrupted. The authors demonstrate that the C2A mutations do not alter the calcium-dependent properties of the native C2A–C2B protein and cause no major changes in synaptic transmission. The authors conclude that calcium binding to the C2A domain of synaptotagmin I is not essential in the presence of a functional C2B domain, and that the two C2 domains are partially redundant in calcium-dependent phospholipid interactions.

39. • Robinson IM, Ranjan R, Schwarz TL: **Synaptotagmins I and IV promote transmitter release independently of Ca^{2+} binding in the C2A domain**. *Nature* 2002, **418**:336-340.
Using transgenic *Drosophila*, this study is the first to demonstrate that mutations in a C2A calcium-binding residue do not result in a null synaptotagmin phenotype. The authors further demonstrate that synaptotagmin IV, an isoform with defective calcium-dependent lipid-binding via its C2A domain, can rescue release defects in synaptotagmin I null mutations.

40. • Mackler JM, Drummond JA, Loewen CA, Robinson IM, Reist NE: **The C2B Ca^{2+}-binding motif of synaptotagmin is required for synaptic transmission *in vivo***. *Nature* 2002, **418**:340-344.
This is the first study to specifically analyze mutations of calcium-binding residues in the C2B domain of synaptotagmin using transgenic *Drosophila*. C2B domain mutants that disrupt calcium-dependent phospholipid binding by the C2B domain *in vitro* have a strong dominant-negative effect *in vivo*, blocking fusion at a post-docking step during neurotransmitter release.

41. • Fernàndez-Chacòn R, Königstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C, Südhof TC: **Synaptotagmin I functions as a calcium regulator of release probability**. *Nature* 2001, **410**:41-49.
This study used knock-in mice to analyze the *in vivo* effects of a mutated arginine residue near the calcium binding loops of C2A; this mutation causes a twofold decrease in calcium-dependent lipid binding of the native protein. Knock-in animals containing the mutant synaptotagmin I show a twofold decrease in release probability as measured in cultured hippocampal neurons. The decrease in release probability is attributed to a decline in the calcium sensitivity of release, as the size of the readily releasable pool and the frequency of miniature EPSCs are unaltered.

42. • Marek KW, Davis GW: **Transgenically encoded protein photoinactivation (FlAsH-FALI): acute inactivation of synaptotagmin I**. *Neuron* 2002, **36**:805-813.
This study describes a new fluorescence photoinactivation technique to acutely disrupt synaptotagmin function at the larval neuromuscular junction in transgenic *Drosophila*. Acute inactivation was accomplished through illumination of a fluorescein derivative coupled to a tetracysteine motif engineered into the C terminus of synaptotagmin I. Physiological recordings done on illuminated animals mimicked defects observed in synaptotagmin loss-of-function mutants, including a large reduction in evoked release and a decrease in calcium cooperativity. Furthermore, the authors use temporally controlled inactivation paradigms to show that the disruption in release is not caused by docking or by endocytotic defects.

43. Littleton JT, Stern M, Perin M, Bellen HJ: **Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in *Drosophila synaptotagmin* mutants**. *Proc Natl Acad Sci USA* 1994, **91**:10888-10892.

44. • Zhang X, Kim-Miller MJ, Fukuda M, Kowalchyk JA, Martin TF: **Ca^{2+}-dependent synaptotagmin binding to SNAP-25 is essential for Ca^{2+}-triggered exocytosis**. *Neuron* 2002, **34**:599-611.
A structure–function characterization of the *in vivo* role of calcium-dependent synaptotagmin/SNAP-25 interactions is described. The authors demonstrate that mutations of several aspartic acid residues (D179, D186, D193) in the C terminus of SNAP-25 disrupt the protein's interaction with synaptotagmin but have little effect on SNARE complex formation. Introduction of mutant SNAP-25 into PC12 cells lacking endogenous SNAP-25 results in only partial rescue of calcium-dependent secretion, suggesting synaptotagmin–SNAP-25 interactions function in large dense-core vesicle exocytosis.

45. Fukuda M, Kabayama H, Mikoshiba K: ***Drosophila* AD3 mutation of synaptotagmin impairs calcium-dependent self-oligomerization activity**. *FEBS Lett* 2000, **482**:269-272.

46. Yamada WM, Zucker RS: **Time course of transmitter release calculated from simulations of a calcium diffusion model**. *Biophys J* 1992, **61**:671-682.

47. • Wang CT, Grishanin R, Earles CA, Chang PY, Martin TF, Chapman ER, Jackson MB: **Synaptotagmin modulation of fusion pore kinetics in regulated exocytosis of dense-core vesicles**. *Science* 2001, **294**:1111-1115.
This study documents changes in the kinetics of the fusion-pore opening of dense-core vesicles in PC12 cells overexpressing synaptotagmin I or synaptotagmin IV. This is the first demonstration that varying the levels of synaptotagmins on dense-core vesicles can alter fusion-pore characteristics and suggests that synaptotagmins function at the final step in fusion. Overexpression of synaptotagmins I and IV had opposite effects on the pore lifetime, with synaptotagmin I functioning to stabilize fusion pores whereas synaptotagmin IV destabilized fusion pores and decreased the number of fusion events.

48. Scales SJ, Finley MF, Scheller RH: **Cell biology. Fusion without SNAREs?** *Science* 2001, **294**:1015-1016.

49. Hsu SF, Augustine GJ, Jackson MB: **Adaptation of Ca^{2+}-triggered exocytosis in presynaptic terminals**. *Neuron* 1996, **17**:501-512.

50. • Rickman C, Davletov B: **Mechanism of calcium-independent synaptotagmin binding to target SNAREs**. *J Biol Chem* 2003, **278**:5501-5504.
In this study the authors demonstrate that native synaptotagmin can bind to the specific t-SNARE heterodimer of syntaxin and SNAP-25, even in the absence of calcium. Synaptotagmin binding requires both C2 domains and both t-SNAREs, as no calcium-dependent interaction was observed with syntaxin and SNAP-25 in isolation. The authors propose that synaptotagmin's interaction with the t-SNARE heterodimer may precede fusion and facilitate SNARE complex formation during the fusion reaction.

51. Ernst JA, Brunger AT: **High resolution structure, stability, and synaptotagmin binding of a truncated neuronal SNARE complex**. *J Biol Chem* 2003, **278**:8630-8636.

52. • Shin OH, Rhee JS, Tang J, Sugita S, Rosenmund C, Sudhof TC: **Sr^{2+} binding to the Ca^{2+} binding site of the synaptotagmin 1 C2B domain triggers fast exocytosis without stimulating SNARE interactions**. *Neuron* 2003, **37**:99-108.
The authors demonstrate that strontium-dependent release (although less robust than calcium-triggered fusion) is impaired in synaptotagmin knockout mice. The authors use biochemical analysis to demonstrate that strontium promotes synaptotagmin-phospholipid interactions but not synaptotagmin-SNARE binding. The authors suggest that the fast phase of release induced by strontium is unlikely to require synaptotagmin-SNARE interactions.

53. Wu Y, He Y, Bai J, Ji SR, Tucker WC, Chapman ER, Sui SF: **Visualization of synaptotagmin I oligomers assembled onto lipid monolayers**. *Proc Natl Acad Sci USA* 2003, **100**:2082-2087.