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Review

# Genetic and molecular analysis of the synaptotagmin family

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**Abstract.** Secretion is a fundamental cellular process used by all eukaryotes to insert proteins into the plasma membrane and transport signaling molecules and intravesicular proteins into the extracellular space. Secretion requires the fusion of two phospholipid bilayers within the cell, an energetically unfavorable process. A conserved repertoire of vesicle-trafficking proteins has evolved that function to overcome this energy barrier and temporally and spatially control membrane fusion within the cell. Within neurons, opening of synaptic calcium channels and subsequent calcium entry triggers synchronous synaptic vesicle exocytosis and neurotransmitter release into the synaptic cleft. After fusion, synaptic vesicles undergo endocytosis, are refilled with neurotransmitter, and return to the vesicle pool for further rounds of cycling. It is within this local synaptic trafficking pathway that the synaptotagmin family of calcium-binding synaptic vesicle proteins has been postulated to function. Here we review the current literature on the function of the synaptotagmin family and discuss the implications for synaptic transmission and membrane trafficking.

**Key words:** Synaptotagmin; neurotransmitter release; exocytosis; synapse; SNARE; endocytosis.

## Overview of membrane fusion

Secretory pathways can be broadly divided into constitutive and regulated modes of membrane trafficking. Constitutive secretion is found in all cell types and is the prominent mode of transport between the endoplasmic reticulum, Golgi, and plasma membrane. Regulated secretion is found primarily in neurons and endocrine cells, where membrane trafficking is finely tuned to intracellular signals such as calcium [1]. The exocytotic machinery in neurons can be viewed as a calcium regulatory system that has been superimposed on a core set of proteins that are utilized in constitutive secretion [for reviews, see refs 2, 3]. Synaptic transmission requires that synaptic vesicles move from a reserve pool to an active cycling pool of vesicles. Synaptic vesicles that are destined to fuse with the presynaptic membrane then undergo docking reactions that position the vesicles in close proximity to the sites of fusion on the plasma membrane. Following docking, the vesicles are further prepared for fusion competence through a series of priming reactions. The molecular events that underlie docking and priming are unknown. However, the protein interactions mediating the last stage in exocytosis, membrane fusion, are beginning to be deciphered.

The core of the membrane fusion machine at synapses (and likely at most, if not all, sites of intracellular fusion) is built around the SNARE complex, composed of the synaptic vesicle protein, synaptobrevin/VAMP, and the plasma membrane proteins syntaxin 1 and SNAP-25 [4]. Large families of these SNAREs are present in all eukaryotes sequenced to date [3] and are distributed in specific membrane compartments along the secretory pathway where they are predicted to function in compartment-specific vesicle fusion within the cell [5]. Structural

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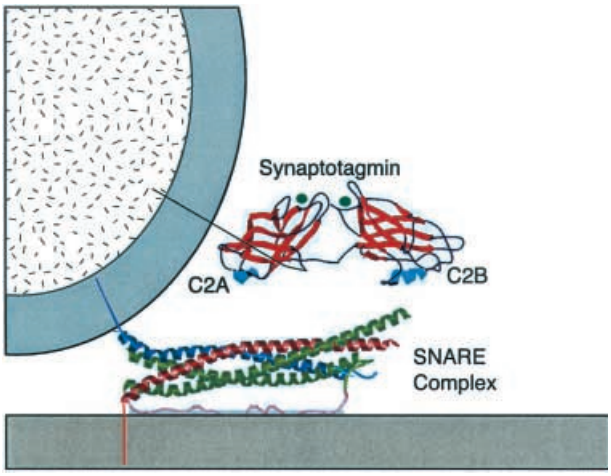


Figure 1. The crystal structure of the SNARE complex [26] and synaptotagmin III [6] shown schematically superimposed on a synaptic vesicle docked at the presynaptic plasma membrane. Transmembrane domains have been added to syntaxin, synaptobrevin, and synaptotagmin. The sites of calcium ion binding to the C2 domains of synaptotagmin are indicated by the green circles. The C2 domains are shown in red (the $\alpha$ helix of each C2 domain is shown in blue). The intravesicular domain of the synaptotagmin domain has been omitted. Synaptobrevin is indicated in blue, SNAP-25 in green, and syntaxin in in red.

analysis has shown that SNAREs assemble into a parallel coiled-coil four-stranded helix with one helix each contributed by syntaxin and synaptobrevin, and two helices contributed by SNAP-25 (fig. l) [6]. The parallel alignment of the helices brings the transmembrane domains of syntaxin and synaptobrevin into close contact, thereby driving the vesicle membrane into close apposition to the presynaptic membrane. Both genetic and biochemical evidence indicate that SNAREs are essential for membrane fusion. Removal of the v-SNARE synaptobrevin in Drosophila results in the elimination of action-potential-evoked vesicle fusion at the neuromuscular junction and a large reduction in the frequency of spontaneous fusion [7, 8]. Similarly, removal of syntaxin 1 from Drosophila eliminates both spontaneous and evoked fusion, without altering the docking of synaptic vesicles at presynaptic active zones [9, 10]. Temperature-sensitive paralytic mutations in Drosophila syntaxin 1 have also been identified that decrease synaptobrevin binding and reduce SNARE complex formation at the paralytic temperature, confirming that v-/t-SNARE interactions are required for neurotransmitter release in vivo [11].

Biochemical evidence indicating that the SNARE complex can mediate bilayer lipid fusion was provided by the work of Rothman and colleagues in in vitro liposome fusion assays. SNAREs are required and sufficient for membrane fusion in this in vitro fusion system [12, 13]. Unlike many in vitro fusion assay paradigms, SNARE specificity in mediating fusion has been demonstrated with several convincing controls. Using biochemically modified SNARE proteins in these assays, formation of the SNARE complex apparently promotes bilayer fusion by exerting force on the membrane anchors of individual SNAREs during SNARE complex formation. This conformational arrangement is likely to lead to inward movement of lipids from the two membranes and eventual lipid mixing [14]. Thus the formation of the SNARE complex has emerged as the central protein machinery that underlies membrane fusion. In addition, specific v-/t-SNARE pairing is likely to underlie compartment specificity in membrane fusion [15]. Modulators of SNARE assembly and disassembly, as well as calcium regulators of SNARE assembly, are now key elements of the fusion machinery that are likely to be experimentally solved over the next decade.

Current studies on synaptic transmission have suggested a complex picture of presynaptic nerve terminal architecture. One of the most defining features of this architecture is the extensive subcellular localization of the fusion machinery. Using electron microscopy, synaptic vesicles can be seen clustered at specific regions on the terminal plasma membrane termed active zones [16, 17]. Active zones, when occupied with synaptic vesicles, are thought to host all the proteins necessary for regulated exocytosis, including presynaptic calcium channels. Electrophysiological studies have begun to elucidate some of the functional consequences of this organization. The maximal rate of calcium-triggered synaptic vesicle fusion can be as rapid as 1000–3000 $s^{-1}$, while the lag time between calcium influx and fusion can be as short as 100 µs [18–20]. Such rapid calcium sensing and membrane fusion suggest that synaptic vesicles are docked and primed prior to calcium influx and that local calcium-induced conformational changes in the exocytotic machinery result in bilayer fusion. This short time lag eliminates enzymatic steps in the final fusion event. Neurotransmitter release has a fourth-order cooperativity with respect to calcium concentration [21], suggesting that multiple calcium ions bind to one or more proteins and/or lipids to trigger vesicle fusion. Current studies suggest synaptic vesicle exocytosis is triggered when the intracellular calcium concentration rises to greater than 20 µM calcium, with half-maximal fusion rates at 194 µM calcium [19]. Such high calcium concentrations are thought to exist only near the pore of presynaptic calcium channels [22], suggesting that the release machinery and calcium channels are co-localized at sites of membrane fusion. The speed of exocytosis and the dynamics of calcium concentrations within the terminal place restrictions on how the fusion machinery can be assembled and function, and point to a small number of protein rearrangements between calcium entry and bilayer membrane fusion.

A mechanistic understanding of how synaptic calcium signals are transduced into membrane fusion is still lacking. However, biochemical and genetic studies on

synaptotagmin I are consistent with the idea that it is a major calcium sensor at synapses, although it likely plays multiple roles in vesicle cycling [for recent reviews, see refs 23, 24]. This conclusion is still debated however, and the precise mechanism by which synaptotagmin I functions is far from elucidated. Currently, 11 synaptotagmin isoforms have been isolated in mammals, and six to eight homologues have been found in *Drosophila* and *Caenorhabditis elegans*. Besides the data obtained for synaptotagmin I, the function of the remaining synaptotagmins is relatively unclear. We will briefly review the literature on the synaptotagmin family and discuss the implications for synaptotagmin function in vesicle cycling.

## Biochemistry of synaptotagmin I

Synaptotagmin I is by far the most studied isoform of the synaptotagmin family and was cloned from rat brain as a 65-kDa synaptic vesicle protein with calcium-dependent phospholipid-binding properties [25]. Synaptotagmin I contains a small N-terminal intravesicular domain, a single transmembrane domain and a large cytoplasmic region (fig. 1). Two domains homologous to the C2 regulatory domain of protein kinase C occupy the majority of the cytoplasmic region of synaptotagmin. C2 domains are ~130-amino acid motifs that are found in a large array of intracellular and extracellular proteins, many of which bind calcium. The crystal structure of several C2-domain-containing proteins, including the cytoplasmic domain of synaptotagmin III and the C2A domain of synaptotagmin I, have been solved, demonstrating that C2 domains fold into an eight-stranded $\beta$ sandwich [26, 27]. Three cytosolic loops surround the calcium-binding ligands and emerge from the core $\beta$ sandwich to form a calcium-binding pocket. The crystal structures indicate that no significant conformational changes occur upon calcium binding. Such observations favor a model of C2 domain function in which changes in surface electrostatic potential mediate many of the calcium-dependent C2 domain interactions. The three loops forming the calcium-binding pocket in the C2A domain of synaptotagmin I insert directly into phospholipid bilayers upon calcium binding [28–30]. The C2A domain of synaptotagmin I has evolved for speed, as it interacts with membranes upon calcium binding with diffusion-limited kinetics, much faster that other C2 domain-lipid interactions that have been characterized [31]. In addition, the EC50 for calcium-dependent phospholipid binding to the C2A domain has been shown to be ~74 µM calcium, approximating the levels of intracellular calcium required to trigger synaptic vesicle fusion [31]. These properties have prompted the hypothesis that the calcium-dependent lipid-binding properties of synaptotagmin I may be involved in lipid rearrangements required during synaptic vesicle fusion.

The C2B domain of synaptotagmin I also binds calcium and undergoes local conformational changes [32]. One consequence of calcium binding by the C2B domain is oligomerization of the protein via clustering by C2B-C2B interactions [30, 33–36]. Calcium-dependent oligomerization of synaptotagmin is extremely rapid, requires calcium concentrations near those required for triggering fusion and is an evolutionarily conserved interaction [31, 35], suggesting that C2B domain-calcium interactions may also function in fusion. Intriguingly, the crystal structure of synaptotagmin III reveals that the linker between the C2A and C2B domains is unstructured and flexible. Fluorescence resonance energy transfer studies have demonstrated that upon calcium binding, both C2 domains undergo an intramolecular interaction that modulates the distance between the two domains [37], suggesting that both domains may also cooperate in calcium-dependent interactions. One interaction known to require both C2 domains together is the ability of synaptotagmin to form calcium-dependent complexes with isolated t-SNAREs and the assembled SNARE complex [31, 38]. Given the central role of SNARE complex formation in fusion, this interaction has also been cited as a potential coupling step in triggering rapid exocytosis. The ability of synaptotagmin to form calcium-dependent complexes with the isolated t-SNAREs syntaxin and SNAP-25 suggests that synaptotagmin may order monomeric SNAREs into helical proteins that could facilitate their incorporation into the SNARE complex. However, synaptotagmin also binds SNAREs and the assembled SNARE complex in the absence of calcium, though with a substantially reduced affinity [31]. Such calcium-independent SNARE interactions could contribute to vesicle docking or perhaps function in a fusion clamp role to prevent SNARE complex formation and vesicle fusion in the absence of calcium. Botulinum A, which cleaves the extreme C-terminal of SNAP-25, disrupts exocytosis in a calcium-dependent fashion. Martin and colleagues have recently demonstrated that botulinum A also specifically blocks the binding of synaptotagmin to SNAP-25 in a calcium-dependent manner, without affecting SNARE complex formation, supporting a role for a calcium-dependent SNAP-25-synaptotagmin interaction in calcium-triggered exocytosis in vivo [38]. The remaining calcium-dependent interactions of synaptotagmin I that have been reported include a reduction in binding to the synaptic vesicle protein SV2 [39] and altered phosphoinositide binding [40]. The significance of these calcium-dependent interactions is unknown.

In addition to calcium-dependent interactions, synaptotagmins have been shown to interact with a host of neuronal proteins in the absence of calcium. These interactions include binding to calcium channels [41–43], sodium channels [44], the clathrin adapter AP-2 [45], the stoned endocytotic proteins [46], $\beta$-SNAP [47], neu-

rexins [48], calmodulin [49], inositol polyphosphates [50], SYNCRIP [51] and polyphosphoinositides [40]. These diverse interactions suggest synaptotagmin I may have important roles in a variety of presynaptic functions, potentially complicating a simplistic view of synaptotagmin as solely a calcium sensor for exocytosis. The interaction of synaptotagmin with two classes of neuronal ion channels, sodium and calcium channels, suggests synaptotagmin may be involved in the presynaptic regulation of ion channel function, though genetic evidence supporting such a role is lacking. The interaction of synaptotagmin with the clathrin adapter AP-2 is evolutionarily conserved in *Drosophila* [35], suggesting a potential role in facilitating endocytosis of synaptic vesicles at nerve terminals. This interaction can be disrupted by binding of the C2B domain of synaptotagmin to the inositol high-polyphosphate series [52], suggesting the potential for modulation of synaptotagmin-AP2 binding. The interaction of synaptotagmin with the putative endocytotic proteins stnA and stnB encoded by the *stoned* locus in *Drosophila* also points towards a role in the modulation of vesicle endocytosis. The interaction of synaptotagmin with $\beta$-SNAP has been postulated to represent a fusion particle, but this interaction is not observed in *Drosophila,* suggesting it is not essential for synaptic vesicle trafficking [35]. Neurexins are polymorphic cell adhesion molecules that have been postulated to be present at synapses. Their interaction with synaptotagmin could play a role in vesicle docking or in localizing synaptic vesicles to subdomains within the nerve terminal. Given the plethora of interactions now reported for synaptotagmin I, genetic dissection of the function of the protein in vivo via the generation of a large collection of specific loss-of-function mutants is clearly required to define which of these interactions actually occur in vivo and what step in vesicle cycling they mediate.

## Functional analysis of synaptotagmin I

Genetic studies have unambiguously demonstrated that disruption of synaptotagmin I leads to profound defects in synaptic transmission, confirming an important role in regulated secretion of synaptic vesicles. To date, genetic evidence indicating a role for synaptotagmin in large dense-core vesicle fusion has not been demonstrated, although synaptotagmins are likely to function in this trafficking pathway as well. However, the calcium dependence of synaptic vesicle and large dense-core vesicle fusion are known to be distinct [19], suggesting that isoforms with distinct calcium-binding properties may function in large dense-core vesicle fusion. Therefore, we will focus our discussion mostly on the role of synaptotagmin in synaptic vesicle fusion. In *Caenorhabditis elegans, synaptotagmin I* mutants are severely defective in muscle movements and show a large reduction in the number of synaptic vesicles at synapses, suggesting defects in endocytosis [53, 54]. Removal of synaptotagmin I in mice disrupts the fast synchronous fusion of synaptic vesicles, without altering asynchronous or latrotoxin-induced fusion, suggesting a role for synaptotagmin in triggering synchronous vesicle release [55]. In *Drosophila, synaptotagmin I* mutants are lethal and show a severe decrease in neurotransmitter release and a large increase in spontaneous vesicle fusion [56–58]. Null mutations do not show a large depletion of synaptic vesicles as seen in *C. elegans,* but rather have a reduced number of docked vesicles and an increase in a population of large-diameter vesicles [59]. Partial loss-of-function mutations that delete the C2B domain show a reduction in the number of synaptic vesicles at photoreceptor synapses under stimulation, suggesting that the C2B domain is required for efficient endocytosis under conditions that drive synaptic recycling [J. T. Littleton et al., unpublished data]. Thus, the loss of synaptotagmin at synapses seems to disrupt, though not eliminate, synaptic vesicle endocytosis, with consequent alterations in vesicle size. Loss of synaptotagmin also alters the ability of synaptic vesicles to be maintained in a fusion-competent state at active zones. One possibility to account for the increase in spontaneous fusion and the decrease number of docked vesicles in *synaptotagmin* mutants is that synaptotagmin functions as a fusion clamp to prevent SNARE-dependent fusion until the arrival of a calcium signal. Loss of this activity in *synaptotagmin* null mutants could account for the increased rate of spontaneous fusion and decrease in the number of docked vesicles.

The defect in endocytosis in *synaptotagmin* mutants also indicates another important point in the interpretation of genetic studies. When studying *synaptotagmin* mutants that alter endocytosis, such defects have to be taken into account and can confound any interpretation of a role for synaptotagmin on the exocytotic side of the vesicle-trafficking pathway. Therefore, partial loss-of-function mutations that do not alter endocytosis are necessary to address the function of synaptotagmin specifically in exocytosis. One such mutant (Y364N) has been found in *Drosophila* that disrupts a highly conserved residue in the C2B domain of synaptotagmin I [58], but which does not interfere with AP-2 binding or disrupt endocytosis in vivo. Instead, this mutation disrupts calcium-dependent oligomerization, resulting in a post-docking defect in vesicle fusion and a failure to assemble SNARE complexes [J. T. Littleton et al., unpublished data]. Thus, the C2B domain is required in vivo for both endocytosis and calcium-dependent fusion following vesicle docking. Functional studies in cracked PC12 cells supports this model, as recombinant C2B domains introduced as dominant negatives can block exocytosis at the final calcium-triggered step in vesicle fusion [32]. Injection of recombinant

C2B domains that have mutations disrupting oligomerizaton of synaptotagmin do not inhibit secretion, presumably by their inability to interact with native synaptotagmin I C2B domains [32]. To date, no mutations disrupting calcium binding by the C2A domain have been reported. The strong conservation of the C2A calcium ligands throughout evolution, however, suggests these residues are also likely to participate in maintaining the efficacy of synaptic transmission, though their precise role awaits further analysis. Taken together, genetic studies demonstrate that bilayer mixing and the fusion reaction itself does not require synaptotagmin I. Rather, synaptotagmin I seems to function by triggering rapid fusion upon calcium influx and mediating efficient and rapid endocytotic retrieval of the synaptic vesicle after fusion.

In addition to genetic studies, many other manipulation studies on synaptotagmin I have been performed. Injection of peptides from the C2 domains of synaptotagmin I into squid giant synapses rapidly and reversibly inhibits neurotransmitter release [60]. Injection of anti-synaptotagmin I C2A domain antibodies into the squid giant synapse blocks evoked release and increases the number of docked synaptic vesicles [61]. This same antibody also blocks calcium-dependent phospholipid binding by the C2A domain. In contrast, injection of anti-synaptotagmin C2B domain antibodies decreases evoked release during repetitive stimulation and also results in a 90% decrease in synaptic vesicle number [62]. Similar findings were observed by antibody injection studies in adrenal chromaffin cells [63]. Together with the results obtained from genetic studies, a simplistic and likely incomplete molecular model underlying the role of synaptotagmin I in trafficking can be summarized as follows. (i) The ability of synaptotagmin to bind SNAREs and other effectors such as neurexins and calcium channels in a calcium-independent fashion results in synaptic vesicles that are positioned and maintained as fusion-competent vesicles at active zones. (ii) The calcium-independent interaction with t-SNAREs maintains the fusion machinery in a fusion-ready state by bringing the t-SNARE complex into close approximation to the v-SNARE synaptobrevin, while preventing premature SNARE complex formation and fusion in the absence of a calcium signal. (iii) Calcium entry triggers synaptotagmin oligomerization via the C2B domain, facilitates SNARE complex assembly, oligomerizes the SNARE complex into a fusion pore and promotes additional local lipid rearrangements via C2A domain-lipid interactions that culminate in rapid and efficient calcium-triggered synchronous vesicle fusion. (iv) Following fusion, synaptotagmin is recognized by the AP-2 adapter proteins and together with the stoned proteins cooperates to ensure rapid endocytotic retrieval of synaptic vesicles back into the recycling pool.

## Diversity of the synaptotagmin gene family

Apart from synaptotagmin I, eleven other synaptotagmins have been identified in mammals (table 1) [see ref 24 for a review]. We have identified eight synaptotagmins in *Drosophila* [3], making the synaptotagmin family one of the largest protein families implicated in membrane trafficking in both invertebrates and vertebrates. Outside of mutations in mouse synaptotagmin IV and overexpression studies on fly synaptotagmin IV, few other genetic manipulations of synaptotagmins have been reported. Thus, the precise role of these additional isoforms is unknown. One striking observation is that most of the synaptotagmin isoforms show calcium-dependent hetero-oligomerization with each other [32, 34, 35], suggesting that hybrid synaptotagmin oligomers with novel calcium sensitivities may represent a unique mechanism for presynaptic plasticity in the nervous system. Indeed, the finding that many of the synaptotagmin isoforms express unique calcium-dependent phospholipid-binding properties and different synaptotagmin-syntaxin calcium affinities [64] suggests that the hetero-oligomers may possess unique trafficking properties [65]. A description of the expression patterns and subcellular localization of each of the isoforms is required, however, to further test this model. Below, we briefly summarize some of the known synaptotagmin family members and their possible functions in membrane trafficking.

Expression pattern analysis, subcellular localization studies, and in vitro biochemical assays have been performed on many of the mammalian family members, while still relatively little is known about others. Two broad subfamilies have been proposed based on expression pattern. The first subgroup, which includes synaptotagmins I–V, X, XI, and Srg 1, is primarily expressed in the nervous system and in other endocrine organs [66–71]. The remaining synaptotagmins (VI–IX) show more ubiquitous expression and are not enriched in the nervous system [64]. In *Drosophila,* synaptotagmins I and IV are expressed abundantly in the nervous system, while synaptotagmins IX and VII are expressed more ubiquitously and are not enriched in the nervous system [35]. The expression pattern of the remaining synaptotagmins is unknown. Surprisingly, not all synaptotagmins are predicted to be found solely on vesicles. Both synaptotagmin III and VI have been found in other cellular compartments, including the plasma membrane [72]. Synaptotagmin VII has been found on lysosomes [73], while synaptotagmin IV has been found not only on synaptic vesicles [34, 35, 74], but also in the Golgi [75, 76]. Synaptotagmin II shows very high homology to its sister isoform, synaptotagmin I, and is likely to be functionally redundant with synaptotagmin I. Synaptotagmin II and I are localized on the same synaptic vesicles in certain areas in rat brain. Furthermore, synaptotagmin I and II can form calcium-de-

Table 1. Synaptotagmin gene family.

| Gene | Species | Expression pattern | Subcellular localization | $Ca^{2+}$-dependent phospholid binding | Oligomerization |
|---|---|---|---|---|---|
| Syt I | human, rat, mouse *Drosophilia, C. elegans* | neuronal | synaptic vesicles | yes | Can form oligomers and heterodimerize with syt II and syt IV |
| Syt II | human, rat, mouse | neuronal, mast cells, pancreatic cells | synaptic vesicles, Iysosomes of mast cells | yes | Can heterodimerize with syt I |
| Syt III | rat, mouse | neuronal, pancreatic cells | presynaptic plasma membrane, secretory granules in $\beta$-cells | yes | Can form Cys-dependent dimers with V, VI, X<br>Can form $Ca^{2+}$-dependent heterodimers with VII |
| Syt IV | rat, mouse *Drosophilia, C. elegans* | neuronal | *Drosophilia* – synaptic vesicles; rat – colocalizes with Golgi markers | no | Can form dimers with syt I |
| Syt V | rat, mouse *Drosophila* (V/X) | neuronal, low expression in rat kidney, adipose tissue, lung, heart | unknown | yes | Can form Cys-dependent dimers with III, VI, X<br>Can form $Ca^{2+}$-dependent heterodimers with VII |
| Syt VI | human, rat, mouse | ubiquitous | rat – somewhat excluded from synaptic vesicles | no | Can form Cys-dependent dimers with III, V, X<br>Can form $Ca^{2+}$-dependent heterodimers with VII |
| Syt VII | human, rat, mouse *Drosophilia, C. elegans* | ubiquitous | unknown | yes | Can form $Ca^{2+}$-dependent dimers with itself and syt III, V, X |
| Syt VIII | human, mouse | ubiquitous | unknown | no | |
| Syt IX | human, rat, mouse *Drosophilia* | ubiquitous | unknown | no | |
| Syt X | rat, mouse | neuronal | unknown | unknown | Can form Cys-dependent dimers with III, V, VI<br>Can form $Ca^{2+}$-dependent heterodimers with VII |
| Syt XI | human, rat, mouse | neuronal | unknown | no | |
| Srg 1 | rat, *Drosphila* | neuronal | unknown | unknown | unknown |

syt, synaptotagmin.

pendent heterodimers [34]. In situ hybridization studies in the rat central nervous system demonstrate that synaptotagmin I and II show a different distribution in the developing nervous system. Northern blot analysis suggests synaptotagmin II is found mainly in the spinal cord while synaptotagmin I transcripts are abundant in brain [77]. Localization studies on synaptotagmin III have been performed both in pancreatic $\beta$-cells and rat neural cells. Butz et al. [72] reported that synaptotagmin III-specific antibodies stain the synaptic plasma membrane fraction and not synaptic vesicles. Localization in the synaptic plasma membrane does not rule out possible functions in vesicle fusion, but may indicate that synaptotagmin III has an important role in other $Ca^{2+}$-mediated presynaptic processes. In pancreatic $\beta$ cells, synaptotagmin III colocalizes with the secretory granule markers, insulin and secretogranin I, suggesting it is found in some vesicle populations as well [78]. Suprisingly, invertebrates lack a synaptotagmin III homolog, suggesting this isoform is likely to subserve specific functions required only in vertebrates. The subcellular localization and function of the remaining synaptotagmins is relatively unknown. One

exception to this is synaptotagmin IV, which is discussed below.

Synaptotagmin IV is upregulated in PC12 cells and hippocampal neurons upon depolarization [74, 79]. It is also upregulated by kainate-induced seizure activity in rat brain. A similar upregulation has been observed for synaptotagmin X [67] and Srg-1 [68], suggesting that modification of expression levels of different synaptotagmin isoforms may have a functional role in neuronal plasticity. One of the most unusual properties of synaptotagmin IV is an evolutionarily conserved amino acid substitution (D to S) in a key calcium-binding ligand in the C2A domain that renders it incapable of calcium-dependent phospholipid binding in *Drosophila* [35]. The calcium-dependent phospholipid binding of rat synaptotagmin IV is controversial and may depend on the phospholipid composition of vesicles [80, 81]. In *Drosophila,* upregulation of synaptotagmin IV can lead to a decrease in both evoked and spontaneous synaptic transmission [35]. In addition, synaptotagmin I/IV hetero-oligomers have reduced calcium-induced phospholipid-binding properties compared to synaptotagmin I/I homo-oligomers [35]. Thus, seizure-induced upregulation of synaptotagmin IV may be a conserved mechanism to downregulate neuronal activity, possibly serving a neuroprotective function. Such an upregulation may also be a mechanism for controlling long-term synaptic plasticity in the brain. Indeed, mutations in mouse synaptotagmin IV lead to defects in both motor performance and learned behavioral outputs [82]. However, there are differences reported in the literature for the subcellular localization of mammalian synaptotagmin IV. Two reports suggest synaptotagmin IV is found on synaptic vesicles [34, 74], while others find the protein in the Golgi and in synaptic regions distinct from synaptotagmin I [75, 76]. Further studies of antibody specificity will be required to resolve the discrepancies in these localization studies. Mammals contain two additional homologs of synaptotagmin IV, synaptotagmin XI [80] and a new synaptotagmin family member KIAA 1342 (GenBank). Further studies of these additional isoforms will be required before the functions of the calcium-independent synaptotagmin isoforms are known.

The recent completion of the Drosophila genome allows us a unique opportunity to examine the entire C2-domain-containing family in a single organism. We have characterized the C2-domain family in flies and identified a large family of C2-domain-containing proteins that may function in membrane trafficking. Figure 2 shows a domain diagram of the C2 family in flies. In yeast, the C2 domain family is small and includes three synaptotagmin-related molecules termed tricalbins, each containing three C2 domains. In addition, one ubiquition ligase/Nedd4-like molecule (Rsp5p), one phosphatidyl serine decarboxylase (Psd2p) and one C2-containing protein of unknown function (296w) can be found. Drosophila contains an expanded repertoire of C2-domain-containing proteins, suggesting novel roles for these proteins in higher metazoans. Among the C2 family are eight proteins most homologous to synaptotagmins. These include homologs of mammalian isoforms I, IV, VII, IX, V/X, VII, Srg-1, and one additional synaptotagmin-like protein. Of these, only the synaptotagmin IX homolog lacks a transmembrane domain. In addition, *Drosophila* con-

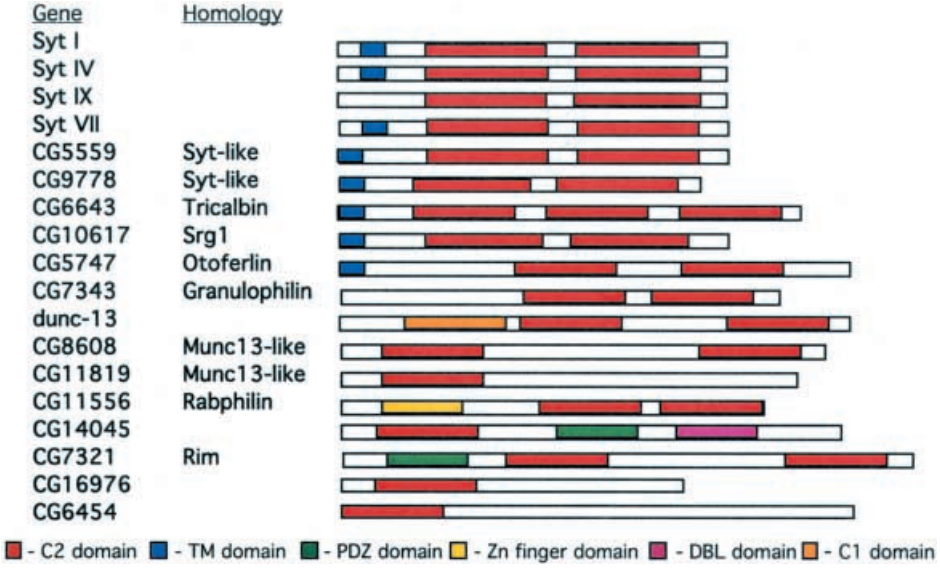


Figure 2. Scheme for the family of C2-domain-containing proteins in *Drosophila.* The transmembrane domain (TM), the C2 domains, and other known motifs found within each protein are also shown (DBL, domain with homology to the dbl/cdc24 rhoGEF family). The CG designation or gene name is shown in the left column and homologies to characterized mammalian proteins are shown in the right column.

tains a number of synaptotagmin-related proteins including one tricalbin homolog, one granulophilin homolog, and one otoferlin homolog. Other C2-domain-containing proteins include one Rabphilin homolog, one Rim homolog, three proteins with some homology to Munc 13, and four additional novel C2-containing proteins. *Drosophila* also contains homologs of PIP kinase, ubiquiton ligases, and protein kinase C. Many of these proteins are conserved in both *C. elegans* and mammals, suggesting specific functions in membrane trafficking. The next major goal is to decipher the calcium-dependent properties of each of these proteins, their subcellular localization, and their role in vesicle trafficking within the cell.

## Conclusion

We have reviewed the current literature on the synaptotagmin family and have focused on the function of synaptotagmin I. Substantial evidence now suggests that synaptotagmin I functions as a calcium sensor for synaptic exocytosis and as an AP-2-binding protein for endocytosis. The precise mechanism by which synaptotagmin tnggers fusion is still being charactenzed. In addition, the role of the majority of the effector interactions of synaptotagmin that have been defined biochemically are still unknown. For the most part, the function of the remaining members of the synaptotagmin family is still a mystery. Solving these puzzles will require a combination of genetics and biochemistry, and will likely continue well into the next decade.


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