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Friends and Foes in Synaptic Transmission—the Role of Tomosyn in Vesicle Priming

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Abstract

Priming is the process by which vesicles become available for fusion at nerve terminals and it is modulated by numerous proteins and second messengers. One of the prominent members of this diverse family is Tomosyn. Tomosyn has been identified as a Syntaxin-binding protein; it inhibits vesicle priming, but its mode of action is not fully understood. Tomosyn's inhibitory activity depends on its N-terminal WD40-repeat domain and is regulated by the binding of its SNARE-motif to Syntaxin. The present review describes new physiological information on Tomosyn's function and addresses possible interpretations of these results in the framework of the recently described crystal structure of the yeast Tomosyn homolog Sro7. We also present possible molecular scenarios for vesicle priming and the involvement of Tomosyn in these processes.

Keywords

Tomosyn; Syntaxin; Priming; SNARE; Synaptic Transmission

Vesicle priming as a key process in synaptic transmission

Synaptic transmission at the nerve terminal involves several steps that lead to the timely and controlled release of neurotransmitter. Following the arrival and docking of a vesicle at the plasma membrane (PM), it undergoes a series of maturation steps that render it fusion-competent. These steps are collectively known as the vesicle priming process and are crucial for vesicle fusion. Priming is coordinated by a series of protein-protein interactions that occur between cytosolic, vesicular and PM proteins[1–3]. A key step in vesicle priming is the formation of multiple heterotrimeric SNARE complexes between the PM SNARE proteins Syntaxin1a and SNAP-25 and the vesicular protein Synaptobrevin/VAMP2[4–8]. Full assembly of the SNARE complex is thought to bring the vesicle into close apposition with the PM and may catalyze the fusion reaction[9]. The priming process is highly regulated by several accessory proteins, such as Munc18, Munc13, Tomosyn, Rabphilin and Complexin. Changes in the levels of some synaptic proteins alter specific steps in synaptic transmission and affect synaptic plasticity processes[10–14]. All of these proteins interact with at least one member of the SNARE protein family. The purpose of this review is to discuss the priming process and the effect of Tomosyn on vesicle priming and synaptic transmission.

Tomosyn

Tomosyn was first discovered as a Syntaxin1a-binding protein in a pulldown assay from rat cerebral cytosol and accordingly, received its Japanese name: *tomo* (“friend” in Japanese) of *syn* (Syntaxin)[15]. It is expressed in the brain and colocalizes with Syntaxin in synapse-

forming regions such as cerebellar molecular layers but it is also found in non-synaptic regions [15]. Tomosyn is partially colocalized with Syntaxin1, Synaptophysin and Bassoon in cultured superior cervical ganglion (SCG)[16] and with Synaptobrevin in *C. elegans*[17], suggesting presynaptic localization. Tomosyn is known as a cytosolic protein but has also been found associated with synaptic vesicles[15,17,18] and other organelles such as insulin-containing granules[19,20], and it also localizes to the PM through its interaction with Syntaxin[21,22]. Tomosyn has three distinct domains (Fig. 1): a C-terminal region containing an R-SNARE, Synaptobrevin-like coiled-coil domain; an N-terminal region enriched with WD40 repeats that are predicted to fold into a propeller-like structure, and a hypervariable linker domain that differs between several splice variants[23–25]. According to recent alignment with Sro7, the yeast Tomosyn homolog, Tomosyn may fold into a twin beta propeller-like structure and the hypervariable linker is predicted to be a part of the C-terminal propeller[26] (Fig. 1).

Two mammalian Tomosyn genes were identified, encoding Tomosyn-1 and Tomosyn-2. Tomosyn-1 is alternatively spliced into three distinct isoforms and Tomosyn-2 has four different splice-variants[23,25]. The s-Tomosyn and m-Tomosyn isoforms are brain-specific [25] and are enriched in synapse-specific domains, suggesting a role in synaptic transmission, while b-Tomosyn is ubiquitously expressed[25] and has been shown to play a role in regulated exocytosis in non-neuronal cell types[22]. The transcription of Tomosyn-2 is steeply upregulated during development: its expression increases 30-fold between E10 and P12, following a pattern similar to Syntaxin and Munc18. mRNA of Tomosyn-1, on the other hand, increases only by a factor of 2.7 during this period[23]. Although the antibodies used in immunohistochemical staining on brain slices cannot distinguish among the isoforms, one can speculate that while Tomosyn-2 is involved in specific restriction of vesicle fusion during synapse formation, Tomosyn-1 has a more general role, acting as a general regulator of axon elongation[23,27]. At the synapses, their expression also differs, suggesting a similar function at different synapses[23]. However, all of the information known to date relates to Tomosyn-1, and the role of Tomosyn-2 is not known.

Physiological effects of Tomosyn

Tomosyn was suggested to displace Munc18 from Syntaxin and form a novel four-protein complex consisting of Syntaxin, SNAP-25 and the calcium sensor Synaptotagmin[15]. Munc18 displacement was thought to release Munc18's inhibition of Syntaxin, and the new four-protein complex was hypothesized to be an intermediate complex, before the formation of the SNARE core complex[15,28]. According to this scenario, Tomosyn would be expected to play a catalytic role in neurotransmitter release. However, in this original study, Tomosyn was already observed to inhibit exocytosis from PC12 cells[15] and recent work has shown that Munc18 does not inhibit exocytosis and is actually required for proper synaptic transmission[29–32]. In addition, Synaptobrevin was not able to displace Tomosyn from the SNARE core complex[24], indicating that if indeed the Tomosyn-SNARE complex is formed under physiological conditions, it may constitute a “dead-end” complex that titrates out functional SNARE proteins[24,33]. Subsequent studies demonstrated that Tomosyn causes a significant reduction in exocytosis in several neurosecretory cells and in neurons[15,20–22, 33–37]. Complementary support came from a series of genetic studies in *C. elegans* that demonstrated that in two different mutants in which *tom-1*, the Tomosyn homolog, was disrupted, neurotransmitter release was enhanced, giving rise to prolonged asynchronous, late release[38,39]. Mechanistically, it was shown that Tomosyn inhibits the priming step in both neuroendocrine cells and neurons[16,17,37,39].

The effects of Tomosyn on vesicle mobility were recently investigated using total internal reflection fluorescence (TIRF) microscopy[36]. Tomosyn overexpression was shown to change the vesicle dynamics near the PM: it attenuated the immobilization of newly arriving

vesicles, which caused an increase in vesicle mobility and a reduction in the time the vesicles remain near the PM. In addition, Tomosyn reduced the release of already-immobile vesicles. These findings suggested that the inhibition of priming is mediated in part by attenuation of the immobilization of newly arriving vesicles, and by a reduction in the release probabilities of docked vesicles[36].

The role of the endogenous mammalian Tomosyn was investigated using silencing techniques and recently by investigating the phenotype of Tomosyn-1 knockout mice. However, these studies yielded conflicting results: according to the notion that Tomosyn inhibits priming and exocytosis, one would predict that deletion of Tomosyn should enhance exocytosis. However, Tomosyn knockdown in SCG neurons reduced synaptic transmission, similar to Tomosyn overexpression in these cells[16]. Likewise, downregulation of Tomosyn reduced insulin secretion from an insulin-secreting INS-1E cell line[19] but enhanced exocytosis in mouse beta-cells[20]. In a recent study the phenotype of the first mouse tomosyn-1 mutant was described. In agreement with the overexpression experiments and the *C. elegans* tom-1 mutants, deletion of Tomosyn-1 in these mice caused enhanced synaptic transmission and reduced pair-pulse facilitation[40]. These data support the hypothesis that Tomosyn has an inhibitory role and deletion of Tomosyn-1 enhances the release probability[37].

It has previously been suggested that Tomosyn plays a positive as well as the established negative role in neurotransmitter release. This is supported by the finding that following intense stimulation, Tomosyn-overexpressing cells exhibited enhanced late secretion: this was evident as an enhanced sustained component during long flash stimulation in chromaffin cells[37,41] and as enhanced asynchronous release during high-frequency stimulation in SCG neurons [16]. It is possible that under high calcium conditions, Tomosyn's inhibitory effect is reduced or that its permissive effects are enhanced. In adipocytes, b-Tomosyn and Munc18c could interact simultaneously with Syntaxin4: this complex has been suggested to prime Syntaxin on the PM, providing further support for a positive role for Tomosyn[22]. Therefore, although Tomosyn is thought to play a negative role, there is some indication of a second, permissive role that still needs to be evaluated.

Tomosyn's structural domains and modes of action

Tomosyn is classified as an R-SNARE protein because the C-terminus of all of its isoforms contains a coiled-coil domain, including 16 fully conserved hydrophobic amino acids and the conserved arginine in layer 0 of the central heptad repeat of the α -helix[24,28,33,42]. This domain shows very high homology to VAMP and serves as the main high-affinity interaction site with Syntaxin1a[24,28,33]. The Tomosyn coiled-coil domain also binds SNAP-23 and Syntaxin4[22,28] and forms a stable core SNARE complex *in vitro* with SNAP-25 and Syntaxin1a[24,33]. It has been suggested that full-length Tomosyn binds both SNAP-25 and Syntaxin1a on the PM only in the presence of its SNARE domain[21].

The Tomosyn/SNAP-25/Syntaxin1a complex is very similar in its biophysical and structural properties to the SNARE complex containing Synaptobrevin and it is formed at a similar rate [19,24,33]. Both complexes disassemble only with the addition of NSF although the Tomosyn complex is not resistant to SDS[24,33] and it is slightly weaker than the complex with VAMP2, as determined by atomic force microscopy[19]. The main difference between the structures of the Tomosyn and neuronal SNARE complexes is found on the surface, where binding of Complexin is not detected in the Tomosyn complex[24].

These findings support the general hypothesis that Tomosyn, when highly abundant, inhibits vesicle fusion through the interaction of its R-SNARE domain with Syntaxin. This interaction forms non-fusogenic SNARE complexes and prevents Synaptobrevin from entering the SNARE complexes. However, a Tomosyn mutant that lacks its SNARE domain[41] or a mutant

that has a reduced affinity for Syntaxin[34] still inhibit exocytosis, albeit to a lesser extent. These data suggest that the SNARE motif is not the sole mediator of Tomosyn's inhibitory activity. Nevertheless, its presence enhances Tomosyn's inhibitory effect following lysophosphatidic acid (LPA) activation (which enhances the interaction with Syntaxin)[21]. Thus, this domain clearly plays a role in Tomosyn inhibition and can boost Tomosyn's activity under certain conditions. This interaction may be modulated by other Tomosyn domains, as the full-length Tomosyn binds Syntaxin more efficiently than the SNARE domain alone[24, 25]. Similarly, the ability of the C-terminal domain of Sro7 to bind to Sec9 (the yeast SNAP-25 homolog) is significantly enhanced by the presence of the N-terminal WD40 region[26,43, 44]. Taken together, this suggests that regions upstream of Tomosyn's SNARE motif are involved in Syntaxin binding[24].

The WD40 motifs present in Tomosyn's N-terminal region (Fig. 1) represent the most highly conserved region among Tomosyn homologs[24]. WD40 repeats consist of a conserved core of approximately 40 amino acids that typically starts with the dipeptides GH (glycine-histidine) and ends with the dipeptides WD (tryptophan-aspartic acid). A stretch of 11 to 24 residues separates adjacent repeat motifs. Each WD repeat forms a single blade in a beta-propeller structure and the number of repeats determines the number of blades in the protein (typically between five to seven repeats)[45,46]. The N-terminal region of Tomosyn shares significant similarity with other WD40-repeat proteins like the *Drosophila melanogaster* protein[47], the mammalian Lgl homolog[48] and Sro7/Sro77 in yeast[44,49] (Fig. 1). Lgl is a tumor-suppressor protein[50] that belongs to a family of cytoskeletal regulators associated with the direction of vesicle transport [51]. Sro7/77 proteins are involved in polarized secretion in yeast and deletion of Sro7/77 blocks exocytosis[44]. These data suggest a role in exocytosis and cell polarity for these homologs.

According to recent alignment with Sro7, Tomosyn was predicted to have up to 14 WD40 repeats[26] and may similarly fold into a double beta-propeller-like structure. This structure would then comprise both the N-terminal part of Tomosyn (already predicted to fold into a propeller-like structure) and the hypervariable linker that may be part of the second, C-terminal beta-propeller (Fig. 1). The contribution of these domains, which constitute 90% of the protein, has not been studied extensively. Recently, disruption of the WD40 domain has been shown to reduce Tomosyn inhibition, both in chromaffin cells expressing a mutant that lacks parts of Tomosyn's N-terminus[41] and in *C. elegans*, in which a tom1 mutant lacking part of its WD40 region, showed enhanced neurotransmitter release[38]. Interestingly, the minimal domain necessary for Tomosyn inhibition includes both the WD40 domain and the hypervariable linker region[41] and this domain is sufficient to target Tomosyn to the synapse[17]. Thus, if Tomosyn is folded into two propellers, both are required for its inhibitory activity, as disrupting either one of them abolishes its inhibitory effects[41].

How does this domain function? Despite the importance of the N-terminal WD40 repeats, the protein-protein interactions of the Tomosyn N-terminus remain elusive. Interestingly, in the *Drosophila* l(2)gl protein, the region responsible for binding to non-muscle Myosin II also possesses a high degree of homology with the WD40-repeat region of Tomosyn[47]. Moreover, the yeast Tomosyn homologs Sro7/77 were shown to bind to yeast Myo1p and Myo2p (equivalent to non-muscle Myosin II and Myosin V in mammalian cells)[49]. Note that Myosin II and V have recently been suggested to be involved in a post-docking step in chromaffin cells and neurons[52–58]. It is therefore possible that the N-terminal domain of Tomosyn interacts with cytoskeletal proteins and modulates exocytosis through these interactions; one possible scenario may be that the N-terminal domain of Tomosyn inhibits exocytosis by interfering with the Syntaxin-Myosin interaction, which is important for exocytosis[56]. It was recently demonstrated that the Tomosyn WD40 domain interacts directly with SNAP-25 and Syntaxin, enhances the oligomerization of SNARE complexes and inhibits synaptic transmission[40].

This study suggested a dual inhibitory role for Tomosyn: both its C-terminal coiled-coil domain and its N-terminal WD40 domain interact with Syntaxin and SNAP-25 and inhibit the formation of productive SNARE complexes: The C-terminal coiled-coil domain by binding to Syntaxin and SNAP-25 and the N-terminal WD40 domain by the formation of SNARE complex oligomers[40].

In Sro7, the N-terminus of the WD40 domain contains a hydrophilic binding site for the C-terminal 'tail' of the protein[26]. It was suggested that Sro7 exists in two conformational states with different affinities for the C-terminal tail. The 'open' conformation has a lower affinity for the C-terminal 'tail', allows binding of the Qbc SNARE region of Sec9, which can regulate its availability to create SNARE complexes. The equilibrium between these states may be affected by other factors that regulate Sro7 activity. Tomosyn appears to retain the regulatory tail, similarly to Sro7, and may possess an equivalent regulatory mechanism that controls Syntaxin or SNAP25 availability and vary according to presence of factors that control the C-terminal tail. Evidence for such intra-molecular regulation was recently reported[40].

Modulation of Tomosyn's interaction and function by second messengers

Tomosyn's interaction with Syntaxin is modulated by several mechanisms. Nicotinic stimulation activates Rho-associated serine/threonine kinase (ROCK) and stimulates the formation of Tomosyn-Syntaxin complexes[27] at the PM[21]. Further activation of ROCK by LPA enhances the inhibitory effect of Tomosyn, and this additional inhibition depends on Tomosyn's SNARE domain[21], suggesting that the SNARE domain-Syntaxin interaction is important for Tomosyn inhibition. RhoA is associated with large dense-core vesicles (LDCVs) and activation of G-protein on the LDCVs inhibits the priming step[59,60]. Therefore, one possible scenario is that this inhibition is mediated through the Syntaxin-Tomosyn complex. Another level of regulation may stem from the activity of NSF. Inhibition of NSF activity enhances the Tomosyn-Syntaxin interaction on the PM[21], but causes displacement of b-Tomosyn from the adipocyte PM[22]. Hence, coordinated activity of NSF and LPA can change the binding and unbinding kinetics of Tomosyn with Syntaxin-SNAP-25 complexes and control Tomosyn's activity[21,22]. The time course of ROCK activation and Tomosyn-Syntaxin complex formation is on the order of minutes[21]. Thus, it is reasonable to assume that this interaction does not affect the release of already-primed vesicles but becomes more significant during intense neuronal activity when it modulates vesicle recruitment and the degree of vesicular pool refilling and can contribute to short-term plasticity.

A third tier of regulation involves Tomosyn's phosphorylation. Tomosyn has been shown to be directly phosphorylated by PKA both *in vivo* and *in vitro*, within the hypervariable linker region at Ser 724[16]. This phosphorylation reduces the interaction between Tomosyn and Syntaxin, possibly contributing to the effects of PKA on synaptic release in neurons. Although non-phosphorylated Tomosyn inhibits release more strongly than a phosphomimetic mutant of Tomosyn, wild-type Tomosyn has an effect similar to that of the phosphomimetic mutant [16], shedding doubt on the functional significance of Tomosyn's regulation by PKA in the synapse (see also Sakisaka et al.[40]). The Lgl protein is phosphorylated by atypical PKC in a region that links the protein's N- and C-terminal regions[61], similar to the PKA phosphorylation site in Tomosyn[16]; this phosphorylation enables an intramolecular interaction between the two halves of the protein and inhibits its interaction with myosin II [61]. Such an auto-inhibitory intramolecular interaction may also occur in Tomosyn, serving as an on/off switch. However, based only on structured-based alignment with Sro7 [26,62], the phosphorylation site is found in a loop within the second WD40 propeller and its phosphorylation might not have the ability to regulate an interaction between the two halves of the protein. Nevertheless, the tail domain of Sro7 has an auto-inhibitory effect on Sro7

binding to Sec9[26] and as the tail is conserved in Tomosyn, similar auto-inhibitory regulation may occur also in Tomosyn.

Mechanisms of docking and priming: interplay between Munc13, Tomosyn and Syntaxin

Recent findings have revealed that manipulations of Munc13, Tomosyn and Syntaxin also considerably affect vesicle docking[14,36,39,63–65]. Deletion of *unc13* or Syntaxin causes a reduction in vesicle docking in *C. elegans*[64,66] and chromaffin cells[63]. Tomosyn knockdown also causes redistribution of synaptic vesicles at the *C. elegans* neuromuscular junction[39], while Tomosyn overexpression enhances the turnover rate of vesicles near the PM in chromaffin cells[36]. How can we explain these data? Munc13 is suggested to stabilize the open form of Syntaxin. In agreement, an *unc13* docking defect is rescued by overexpression of an open form of Syntaxin, suggesting that an open Syntaxin molecule is needed for vesicle docking, as well as for vesicle priming[67] in some synapses. Furthermore, the morphological phenotype of *unc13* mutant animals is also rescued by crossing with a *Tom-1* mutant[66]. However, functionally, these synapses are only partially rescued and only crossing *unc13* and *tom-1* mutants with an open Syntaxin mutant improves animal mobility compared to the *unc13* and open Syntaxin mutant[17]. These findings place Syntaxin in a central position in both vesicle docking and priming, and Tomosyn and *unc13* may act through Syntaxin in an antagonistic manner. These data also suggest that docking and priming are molecularly interlinked and can be regarded as successive steps in the protein-protein interactions[68], leading to the formation of assembled SNARE complexes and fusion[14].

Mechanistically, if equilibrium between Munc13 and Tomosyn exists[14], any perturbation of this homeostasis will change the availability of Syntaxin. Thus, in the absence of *unc13*, Tomosyn can prevent Syntaxin from forming SNARE complexes. Accordingly, Munc13 may prevent Tomosyn from forming complexes with SNARE proteins, or alternatively, it may facilitate the disassembly of the non-fusogenic Tomosyn-SNARE complexes. Moreover, the absence of Tomosyn causes accumulation of *unc13* at release sites[17]. Hence, changes in Tomosyn expression levels may constitute a mechanism that modulates release probability and synaptic strength. A caveat to this assumption is that the level of Tomosyn is only 3% of that of Syntaxin[15] and under physiological conditions, it seems unlikely that this low level could have such a profound effect. These findings can be reconciled by assuming that Tomosyn is concentrated in the active zone and at release sites (for example, in hot spots or Syntaxin clusters; Fig 2)[69], similar to the accumulation of Syntaxin and Tomosyn in growth cones [27].

Tomosyn's possible modes of action

Tomosyn can be envisioned as functioning in two steps: the Tomosyn SNARE motif could serve as a spatial signaling marker recruiting Tomosyn to specific sites on the PM that are enriched with Syntaxin, and this interaction itself could inhibit SNARE-complex formation and priming (Fig. 2b). Then, the WD40 domain with the hypervariable linker region, either through oligomerization of cis-SNARE complexes or via interaction with Myosin/ cytoskeleton, could further control Tomosyn's inhibition at the PM (Fig. 2d). Similarly, Hattendorf et al. suggest that Sro7 interaction with the cytoskeleton is coupled to displacement of the C-terminal tail from its N-terminal binding site[26]. In this partially open conformation, Sro7 could both recruit Sec9 to the fusion sites at the membrane and inhibit binding to other SNAREs[26].

Tomosyn can affect both vesicle priming and turnover if we assume that it regulates the transition of morphologically docked vesicles into a functionally docked state (Fig. 3): Vesicles

undergo morphological docking or tethering through interactions with subcortical cytoskeleton or other proteins, such as the exocyst complex [70] or Munc18[31] (Fig. 3, step 1). At this stage, their mobility near the PM is still high. Once a vesicle has formed a single trans-SNARE complex, which represents the first step in the priming process, its mobility decreases (Fig. 3, step 2). Additional SNARE complexes are formed once the vesicle is immobilized, progressing vesicle priming, without affecting its mobility[36] (Fig. 3, step 3). Tomosyn could interfere with steps 2 or 3, both representing the formation of SNARE complexes[36]. Interfering with the formation of the first SNARE complex (Fig. 3, step 2) would attenuate vesicle immobilization and increase the turnover rate of newly arriving vesicles. Interfering with the formation of subsequent SNARE complexes (Fig. 3, step 3) would decrease the release probability of already immobile, resident vesicles[36]. Hence, under Tomosyn overexpression, vesicles are bound by fewer SNARE complexes and are therefore less fusion-competent.

Tomosyn possesses multiple functional domains that can regulate its activity. The interaction with Syntaxin through its SNARE domain is dynamically regulated. We can speculate that, as the surface of the Tomosyn-Syntaxin-SNAP-25 complex differs from that of the Synaptobrevin-Syntaxin-SNAP-25 SNARE complex, it can interact with other proteins involved in exocytosis[24], providing another tier of regulation. The entire N-terminal WD40 domain and linker form an integral functional domain that is essential for Tomosyn's inhibitory activity[41] and may fold into a twin beta-propeller structure, similar to Sro7[26]. This domain could interact with cytoskeletal elements[47,49] or with as-yet unidentified synaptic proteins (e.g., exocyst protein Exo84 has been shown to interact with Sro7/77[70]), and may also regulate the Syntaxin-Tomosyn interaction[23,24,26,33,41] by serving as an intramolecular switch. In addition, the WD40 repeats can control SNARE complex oligomerization and provide another level of regulation of synaptic transmission. The ability of Tomosyn to enhance the formation of SNARE oligomers as part of its inhibitory activity suggest that these oligomers should be non productive, probably cis-SNARE complexes. Hence both its SNARE motif interaction with Syntaxin and its WD40 repeat-induced SNARE oligomerization, act to reduce the availability or the formation of trans SNARE complexes that are essential for fusion. We are still lacking essential information whether other proteins, beside the SNARE proteins, interact with Tomosyn and how they affect its activity. Finally, we need to gain a better understanding of the exact molecular mechanisms by which Tomosyn operates and its involvement in various synaptic plasticity processes and whether Tomosyn-1 and Tomosyn-2 act similarly. Nevertheless, it is clear that Tomosyn functions at a central stage of the vesicle maturation process in concert with Munc13 and Syntaxin and can fine-tune synaptic strength via various types of stimulation

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Figure 1. Domain structure of Tomosyn and its homologs

The N-terminus of all Tomosyn isoforms is enriched with WD40 repeats that are predicted to fold into an N-terminal propeller-like structure and a C-terminal propeller-like structure (light green boxes). The C-terminus of all Tomosyn isoforms contains an R-SNARE, Synaptobrevin-like coiled-coil domain (red box). Mouse m-Tomosyn is shown as a representative of all Tomosyn isoforms. A SNARE-like domain exists in Sro7 and in L(2)gl (dark blue box). A hypervariable domain (HVR) is part of the putative C-terminal propeller-like structure (dark green box) and varies in length between the different splice variants (only the m-Tomosyn isoform is shown here). The PKA phosphorylation site in Tomosyn and the 3 aPKC phosphorylation sites in L(2)gl are labeled with P. Sro7 has two beta propeller structures (green boxes). The first and the last amino acids are labeled for each of the proteins.

Figure 2. A putative model for the inhibitory activity of Tomosyn under resting conditions and after stimulation

(a) Under resting conditions, a docked, unprimed vesicle forms trans-SNARE complexes via interaction of its membrane-bound VAMP with PM-bound Syntaxin and SNAP-25 to form multiple SNARE complexes. This process renders the vesicle fusion-competent (primed) and it can then fuse upon elevation of $[Ca^{2+}]_i$; (b) Tomosyn is recruited through its SNARE motif to areas on the PM enriched with Syntaxin molecules and inhibits SNARE complex formation. (c) After intense stimulation, ROCK phosphorylates Syntaxin at Ser14 and this increases the interaction between Tomosyn and phosphorylated syntaxin, and a greater number of Tomosyn proteins are recruited to the membrane through binding to phosphorylated Syntaxins. (d) Dual

inhibition by Tomosyn. In addition to the Syntaxin-Tomosyn interaction, the WD domain enhances oligomerization of cis-SNARE complexes and further reduces vesicle priming. The WD domain may also interact with the cytoskeleton. Thus, under Tomosyn overexpression or upon specific stimulation, Tomosyn can impose greater inhibition by forming complexes with SNAP-25 and Syntaxin or by enhancing oligomerization of cis-SNARE complexes, limiting the amount of uncomplexed PM-bound SNAREs and therefore reducing the formation of trans-SNARE complexes. For clarity, only the SNARE domain of Syntaxin is shown.

Figure 3. Schematic representation of vesicle docking and priming, and Tomosyn's effects on vesicle mobility and turnover

Vesicles undergo morphological docking or tethering through interactions with the subcortical cytoskeleton or with other proteins, such as the exocyst complex or Munc18 (step 1). Following the formation of the first trans-SNARE complex, the vesicle's mobility decreases (step 2). This represents the first step in the priming process and the formation of a functional docked vesicle. Additional SNARE complexes are formed once the vesicle is immobilized, without affecting its mobility (step 3). Upon binding of accessory proteins such as Complexin and/or Synaptotagmin, priming is complete and the vesicle becomes fusion-competent (step 4). Note that the priming process consists of several steps until the vesicle becomes fully fusion-competent. Steps 2 and 3 involve an identical molecular process, the formation of trans-SNARE complexes, although step 2 involves a decline in vesicle mobility and step 3 does not. Tomosyn can interfere with the formation of SNARE complexes in steps 2 or 3. Interfering with step 2 will increase the turnover rate of newly arriving vesicles and inhibit their immobilization. Interfering with step 3 will reduce the fusion-competence of the vesicles and decrease its release probability.