Tomosyn Inhibits Synaptic Vesicle Priming in Caenorhabditis elegans

Elena O. Gracheva, Anna O. Burdina, Andrea M. Holgado, Martine Berthelot-Grosjean, Brian D. Ackley, Gayla Hadwiger, Michael L. Nonet, Robby M. Weimer, Janet E. Richmond

1 Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois, United States of America, 2 Biology Department, Loyola University Chicago, Chicago, Illinois, United States of America, 3 Department of Anatomy and Neurobiology, Washington University School of Medicine, Saint Louis, Missouri, United States of America, 4 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, United States of America

Caenorhabditis elegans TOM-1 is orthologous to vertebrate tomosyn, a cytosolic syntaxin-binding protein implicated in the modulation of both constitutive and regulated exocytosis. To investigate how TOM-1 regulates exocytosis of synaptic vesicles in vivo, we analyzed C. elegans tom-1 mutants. Our electrophysiological analysis indicates that evoked postsynaptic responses at tom-1 mutant synapses are prolonged leading to a two-fold increase in total charge transfer. The enhanced response in tom-1 mutants is not associated with any detectable changes in postsynaptic response kinetics, neuronal outgrowth, or synaptogenesis. However, at the ultrastructural level, we observe a concomitant increase in the number of plasma membrane-contacting vesicles in tom-1 mutant synapses, a phenotype reversed by neuronal expression of TOM-1. Priming defective unc-13 mutants show a dramatic reduction in plasma membrane-contacting vesicles, suggesting these vesicles largely represent the primed vesicle pool at the C. elegans neuromuscular junction. Consistent with this conclusion, hyperosmotic responses in tom-1 mutants are enhanced, indicating the primed vesicle pool is enhanced. Furthermore, the synaptic defects of unc-13 mutants are partially suppressed in tom-1 unc-13 double mutants. These data indicate that in the intact nervous system, TOM-1 negatively regulates synaptic vesicle priming.

Introduction

Membrane fusion is mediated by the interactions of cognate SNARE (soluble NSF attachment protein receptor) proteins associated with vesicle and target membranes [1,2]. Synaptic vesicle exocytosis is a highly specialized form of membrane fusion in which calcium triggers fusion of synaptic vesicles with the plasma membrane, resulting in neurotransmitter release. Prior to vesicle fusion, the plasma membrane Q-SNAREs syntaxin-1a and SNAP-25 assemble with the vesicle-associated R-SNARE synaptobrevin-2 (a.k.a. VAMP-2) to form a stable coiled-coil complex known as the SNARE complex [3,4]. The assembly of the SNARE complex in trans is thought to bring the vesicle into close apposition with the plasma membrane, and may drive the fusion reaction [5]. Several synaptic proteins have been implicated in the regulation of this fusion process through their SNARE interactions, including the recently identified protein tomosyn [6].

Tomosyn is a 130 kDa soluble protein first isolated from rat cerebral cytosol as a syntaxin-binding partner capable of disrupting Munc18–syntaxin-1a complexes [6]. There are two paralogous tomosyn genes in the mammalian genome (tomosyn-1 and –2) that give rise to seven tomosyn isoforms through differential splicing [7,8]. All mammalian tomosyn isoforms have two recognizable domains, an N-terminal domain rich in WD40 repeats and a C-terminal SNARE domain with high sequence homology to the R-SNARE domain of synaptobrevin [9,10]. WD40 repeats are known to form beta propellers that act as protein interacting modules, although binding partners of the tomosyn WD40 repeats have yet to be identified. The tomosyn R-SNARE domain interacts with syntaxin and SNAP-25 to form a tomosyn SNARE complex that does not contain synaptobrevin but still binds to the putative calcium sensor synaptotagmin [6]. The biophysical properties of the tomosyn SNARE complex resemble those of the SNARE complex; both form at similar rates, have strong hysteresis during folding/unfolding transitions, exhibit alpha helicity, and are disassembled by NSF [10]. Consistent with these properties, the crystal structure of the core tomosyn SNARE complex reveals a four-alpha helical arrangement between the SNARE domains of tomosyn, SNAP-25, and syntaxin that is similar to the structure of the synaptobrevin-containing SNARE complex [11]. As predicted from the similarity in crystal structures, synaptobrevin is unable to displace tomosyn from the tomosyn SNARE complex (and vice versa), without prior NSF disassembly [11]. These data imply that formation of tomosyn SNARE complexes may preclude synaptobrevin-containing SNARE complex assembly, and therefore negatively regulate vesicle exocytosis. Consistent with this model, overexpression of vertebrate tomosyn reduces depolarization-induced dense-plaque formation.

Academic Editor: Fred Hughson, Princeton University, United States of America
Received March 3, 2006; Accepted June 6, 2006; Published July 25, 2006
DOI: 10.1371/journal.pbio.0040261
Copyright: © 2006 Gracheva et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Abbreviations: EM, electron microscopy; GFP, green fluorescent protein; NMJ, neuromuscular junction; RNAi, RNA interference; SNARE, soluble NSF attachment protein receptors; TOM-1Ct, C-terminal coiled-coil motif of TOM-1; WT, wild type

* To whom correspondence should be addressed. E-mail: jer@uic.edu
core granule fusion from PC12 cells [6,10], chromaffin cells [12], insulin-mediated exocytosis of GLUT4-containing vesicles from adipocytes [13], insulin release from beta cells [14], and synaptic transmission in cultured superior cervical ganglion neurons [15]. Although these data support a negative regulatory role for tomosyn in vesicle fusion, tomosyn RNA interference (RNAi) experiments have yielded mixed results. In cultured superior cervical ganglion neurons, tomosyn RNAi inhibited evoked release [15], whereas tomosyn RNAi in mouse beta cells enhanced exocytosis [14].

To address the role of tomosyn in synaptic transmission, in this study we directly assayed the physiological phenotype of tomosyn loss-of-function mutants at the Caenorhabditis elegans neuromuscular junction (NMJ). Our results indicate that tomosyn inhibits synaptic transmission through actions that regulate the size of the readily releasable vesicle pool.

**Results**

**tom-1 Encodes the C. elegans Tomosyn Homolog**

The C. elegans genome encodes a single tomosyn gene [16], tom-1, that shares significant identity (~33%) with isoforms of mammalian tomosyn-1 and tomosyn-2 (Figure 1A). Like vertebrate tomosyn, C. elegans tom-1 encodes multiple alternatively spliced isoforms, TOM-1(A,B,C) which share the C-terminal coiled-coil motif resembling the R-SNARE domain of C. elegans synaptobrevin (SNB-1) (Figure 1B). The TOM-1B isoform is much smaller than either TOM-1A or TOM-1C, and lacks the N-terminal WD40 repeats. To examine whether the common C-terminal coiled-coil motif of TOM-1 (TOM-1Ct) interacts with syntaxin and SNAP-25, we performed in vitro pull-down assays using recombinant C. elegans proteins. We compared the behavior of synaptobrevin and TOM-1Ct in complex assembly assays with syntaxin-GST (UNC-64) [17] and SNAP-25 (RIC-4) [18]. Both SNB-1 and TOM-1Ct formed complexes, but did so inefficiently (Figure 1C). Systematic replacement of each protein in the SNARE complex assay revealed that all assays containing the C. elegans SNAP-25 formed inefficiently, while all other mixed species formed complexes efficiently (Figure S1). When the TOM-1Ct was mixed with C. elegans syntaxin-GST and vertebrate SNAP-25, syntaxin-GST co-precipitated TOM-1Ct and SNAP-25 (Figure 1D). TOM-1Ct did not form a stable binary complex with syntaxin (Figure S2). TOM-1Ct formed complexes as efficiently as SNB-1 forms complexes, but the TOM-1-containing complex was SDS sensitive, while the SNB-1-containing complex was SDS resistant. Density traces of the Coomassie blue-stained protein incorporated into the mixed-species TOM-1 complex gave densitometry ratios of 1:1:3:1 when divided by the molecular mass for each of the fusion proteins (Figure 1D), consistent with the 1:1:1 stoichiometry reported for the vertebrate tomosyn complex [10]. These data confirm that C. elegans TOM-1 has the ability to form pseudo-SNARE complexes similar to those of vertebrate tomosyn.

**tom-1 Mutant Behavioral and Electrophysiological Phenotypes**

To investigate the function of TOM-1 in vivo, we characterized the phenotypes of two previously isolated tomosyn hypomorphic mutants, tom-1(ok285) and tom-1(nu468), which disrupt both full-length TOM-1 isoforms (TOM-1A/C) but are not predicted to disrupt the short TOM-1B isoform (Figure 1A) [16]. These mutants are viable but exhibit hypersensitivity to the acetylcholinesterase inhibitor aldicarb, suggesting that cholinergic neurotransmission is enhanced either pre- or postsynaptically [16]. In several behavioral assays tom-1 mutants exhibit mild defects, including reduced brood size (331.5 ± 7.2 progeny, n = 10, for wild-type [WT] vs. 299.2 ± 14.4 progeny, n = 9, for tom-1(ok285), p = 0.055; and 206.6 ± 11.8 progeny, n = 9, for tom-1(nu468), p < 0.0001), decreased thrashing rates in solution (121.7 ± 1 body bends/min, n = 10, for WT vs. 103.2 ± 3.9 body bends/min, n = 10, for tom-1(ok285), p = 0.0092; and 92.3 ± 2.4 body bends/min, n = 10, for tom-1(nu468), p < 0.0001), and altered responses to head taps: specifically, tom-1 mutants exhibited increased forward/backward reversals (1.3 ± 0.4 reversals, n = 7, for WT vs. 4.9 ± 1.5 reversals, n = 7, for tom-1(ok285), p = 0.036; and 4.1 ± 0.9 reversals, n = 7, for tom-1(nu468), p < 0.01) and increased incidence of pauses (0 pauses, n = 7, for WT vs. 4.4 ± 1.5 pauses, n = 7, for tom-1(ok285); and 7.3 ± 2.2 pauses, n = 7, for tom-1(nu468)). tom-1 mutants were indistinguishable from WT for other behaviors, such as defecation cycle (intervals between expulsion events: 53.4 ± 0.5 s, n = 5, for WT vs. 53.2 ± 2 s, n = 5, for tom-1(ok285); and 52.8 ± 1.3, n = 6, for tom-1(nu468)). The aldicarb hypersensitivity and behavioral changes of tom-1 mutants are consistent with the hypothesis that TOM-1 function is required for proper signaling between neurons.

To directly test whether mutations in tom-1 alter synaptic transmission, we recorded synaptic responses from the NMJs of dissected worms. Evoked responses were elicited by applying a depolarizing stimulus to the ventral nerve cord and recorded from voltage-clamped postsynaptic body wall muscles. In 5 mM Ca2+ saline the evoked current amplitudes were not significantly altered in tom-1 mutants (2.327 ± 107 pA, n = 47, for WT vs. 2.443 ± 136 pA, n = 37, for tom-1(ok285), p = 0.5; and 2.560 ± 283 pA, n = 11, for tom-1(nu468), p = 0.37) (Figure 2A and 2B). However, the charge integral, a measure of the total ion flux during the evoked response (18.1 ± 0.97 pC, n = 49, for WT vs. 38.2 ± 3.7 pC, n = 30, for tom-1(ok285), p < 0.0001; and 42.6 ± 5.6 pC, n = 8, for tom-1(nu468), p < 0.0001) (Figure 2B), was greatly increased in tom-1 mutants due to a prolonged postsynaptic response (half-time-evoked decay: 4.6 ± 0.17 ms, n = 47, for WT vs. 12 ± 1.1 ms, n = 30, for tom-1(ok285), p < 0.0001; and 12.3 ± 1.7 ms, n = 8, for tom-1(nu468), p < 0.0001) (Figure 2B). The enhanced evoked charge integral of tom-1 mutants in the absence of an increase in evoked amplitude could reflect saturation of the postsynaptic receptor field under the relatively high calcium (5 mM) recording conditions used. To test this possibility, we measured the evoked responses of tom-1 mutants in 0.5 mM Ca2+. In lower calcium, the evoked response amplitudes of tom-1 mutants were still comparable to the WT (1,100 ± 108 pA, n = 13, for WT vs. 1,140 ± 178 pA, n = 7, for tom-1(ok285), p > 0.1; and 1,533 ± 202 pA, n = 8, for tom-1(nu468), p > 0.05); however, the response duration remained significantly prolonged (half-time decay: 2.7 ± 0.17 ms for WT vs. 4.2 ± 0.57 ms for tom-1(ok285), p = 0.016; and 4.2 ± 0.37 ms for tom-1(nu468), p = 0.0041), again resulting in increased charge integrals (6.9 ± 0.82 pC for WT vs. 10.7 ± 1.2 pC for tom-1(ok285), p = 0.018; and 13.3 ± 2 pC for tom-1(nu468), p = 0.006). These data suggest that the enhanced release observed in tom-1 mutants is due primarily to a prolongation of the evoked response.
(A) Gene structure of the three *C. elegans* TOM-1 isoforms. All isoforms were confirmed by expressed sequence tags 3’ to the 6th exon and the upstream 5’ region common to TOM-1A, and TOM-1C was determined by 5’ RACE. The two long isoforms TOM-1A and TOM-1C contain N-terminal WD40 repeats and a C-terminal SNARE domain. The short isoform (TOM-1B) contains only the SNARE domain. *tom-1(nu468)* is a G to A change in W212 resulting in an early stop predicted to disrupt isoforms TOM-1A and TOM-1C [16]. The mutation in *tom-1(ok285)* is a 1,580-bp deletion that removes part of exon 10 and all of exons 11 through 13. *tom-1(ok285)* disrupts TOM-1A but, by RT-PCR can produce a mRNA capable of encoding an alternative isoform of TOM-1C lacking exons 11–13 and containing a partial exon 10 and an extra eight amino acids.

(B) Amino acid alignment of the R-SNARE domain of rat tomosyn-1, mouse tomosyn-2, *C. elegans* TOM-1, and *C. elegans* synaptobrevin-1. Identity is shown as black boxes and similarity as gray boxes. Numbers below indicate helical layers formed during SNARE complex assembly.

(C) TOM-1Ct forms tomosyn SNARE complexes with syntaxin and SNAP-25 in vitro to the same extent as *C. elegans* synaptobrevin (SNB-1) forms synaptobrevin SNARE complexes. *C. elegans* syntaxin::GST, or GST alone was incubated with SNAP-25–His6 and either His6T7-tagged TOM-1Ct, or His6T7-tagged SNB-1. Complexes were isolated using glutathione agarose beads. SDS resistance of complexes was assayed by heating half of the pulldown before electrophoresis. Proteins were separated on SDS-PAGE and transferred to nitrocellulose and detected with anti-GST, anti-His6, and anti-T7 antibodies.

(D) Mixed-species tomosyn SNARE and synaptobrevin SNARE complexes form efficiently. Complexes were formed and isolated as described for (C) except that rat SNAP-25 (vSNAP-25–His6) was used in place of the *C. elegans* protein. Complexes were visualized by Coomassie Blue staining after SDS-PAGE. Complex stoichiometry of the tomosyn SNARE complex (*) was estimated by densitometry shown on left. The integrated density of each peak is listed. The molecular weight markers are (from top to bottom) 97 kDa, 66 kDa, 45 kDa, 31 kDa, 21 kDa, and 14 kDa.

DOI: 10.1371/journal.pbio.0040261.g001

Figure 1. TOM-1 Is the *C. elegans* Tomosyn Homolog

PLoS Biology | www.plosbiology.org August 2006 | Volume 4 | Issue 8 | e261
Although the TOM-1A expression experiments indicate that enhanced release in tom-1 mutants has a presynaptic origin, the prolonged duration of the evoked response in tom-1 mutants could be due to altered postsynaptic receptor kinetics. We therefore examined the electrophysiological properties of miniature postsynaptic events (Figure 2C and 2D). Neither the decay rates (decay half-width: 2.05 ± 0.05 ms, n = 57, for WT vs. 2.03 ± 0.07 ms, n = 25, for tom-1(ok285), p > 0.5; and 2.12 ± 0.12 ms, n = 15, for tom-1(nu468), p > 0.8), the amplitude (24.7 ± 0.8 pA, n = 57, for WT vs. 22.4 ± 0.8 pA, n = 25, for tom-1(ok285), p = 0.1; and 26.8 ± 2.3 pA, n = 16, for tom-1(nu468), p = 0.28), nor the frequency (94.3 ± 6.6 Hz, n = 57, for WT vs. 88.4 ± 8.9 Hz, n = 26, for tom-1(ok285), p = 0.6; and 10.3 ± 13.5 Hz, n = 16, for tom-1(nu468), p = 0.6) (Figure 2C and 2D) of miniature synaptic events in tom-1 mutants were significantly different from the WT. These results suggest that the tom-1 mutant synaptic phenotype is not due to changes in postsynaptic reception. Consistent with this conclusion, the miniature postsynaptic response kinetics of the neuronal TOM-1A–expressing integrants (jaIs1052) (decay: 2.46 ± 0.2 ms, n = 7, p = 0.14; minifrequency: 84.8 ± 12.7 Hz, n = 8, p = 0.45) were not significantly different from tom-1(nu468) alone, despite the rescue of the evoked response duration and charge integral (Figure 2C and 2D).

Neuronal Architecture of tom-1 Mutants

Vertebrate tomosyn has been implicated in the regulation of neurite outgrowth in mammalian cultured neurons [19]. Therefore, we next addressed whether the enhanced evoked release in C. elegans tom-1 mutants could be due to aberrant neuronal connectivity. To examine the neuronal cytoarchitecture of tom-1 mutants, we crossed in an integrated array (jaIs14) expressing cytoplasmic green fluorescent protein (GFP) under the cholinergic neuron specific promoter Pacr-2 [20]. Analysis of axonal left/right orientation, axon targeting, and axon fasciculation revealed no differences in the neuronal morphology of tom-1(ok285)jaIs14 relative to the WT (Figure 3A and 3B), suggesting that the tom-1 phenotype is not associated with any discernable innervation defects.

To test for possible changes in synaptogenesis, we examined the number and distribution of neuromuscular synapses in tom-1 mutants. C. elegans neuromuscular synapses, which form in passant along the ventral nerve cord onto body wall muscles, can be visualized using pre- and postsynaptic markers. Specifically, synapses in WT and tom-1 mutant animals were immunolabeled with antibodies to the presynaptic vesicular ACh transporter, UNC-17 [21] (Figure 3C), and UNC-29, a postsynaptic muscle ACh receptor subunit (Figure 3D). Analysis of the staining revealed that the number of synapses based on presynaptic staining (3.13 ± 0.1 UNC-17 puncta/10 μm, n = 5, for tom-1(nu468) vs. 3.6 ± 0.27 puncta/10 μm, n = 5, for WT, p = 0.14) and postsynaptic staining (2.89 ± 0.20 UNC-29 puncta/10 μm, n = 9, for tom-1(nu468) vs. 3.08 ± 0.24 puncta/10 μm, n = 10, for WT, p > 0.5) was not significantly altered. Similarly, the size of presynaptic puncta (1.35 ± 0.06 μm, n = 5, for tom-1(nu468) vs. 1.5 ± 0.1 μm, n = 5, for WT, p = 0.69) and postsynaptic puncta (1.61 ± 0.08 μm, n = 5, for tom-1(nu468) vs. 1.74 ± 0.07 μm, n = 5, for WT, p = 0.19) were not significantly affected in tom-1(nu468). The reduction in evoked response in the TOM-1A–expressing strain (jaIs1052) could also not be attributed to discernable changes in the number or size of cholinergic neuromuscular
synapses (Figure 3). These data suggest that the tom-1 phenotype is not associated with any overt defects in morphogenesis of the nervous system and might therefore reflect a specific defect in synaptic function.

Ultrastructural Analysis of tom-1 Mutants

To test whether the enhanced release in tom-1 mutants resulted from increased synaptic vesicle biogenesis or altered vesicle distribution within synapses, we examined synaptic ultrastructure by electron microscopy (EM) (Figure 4). Recent advances in high-pressure freeze and freeze substitution fixation techniques to prepare worms for EM have greatly improved the quality of ultrastructural data obtained in C. elegans [22]. The advantage of this technique over conventional fixation is that by virtue of the instantaneous freezing of the worms and gradual substitution with fixative, there is less fixation-induced osmotic shock that normally results in cell shrinkage and possible redistribution of synaptic vesicles to the plasma membrane. Using this technique, we found that while the average number of vesicles per synaptic profile did not differ from the WT in tom-1(ok285) mutants (17.45 ± 0.86 vesicles/profile, n = 86 profiles, for WT vs. 17 ± 0.81 vesicles/profile, n = 74 profiles, for tom-1(ok285)), vesicle localization was affected. Specifically, tom-1(ok285) mutants exhibited a dramatic and significant (p < 0.0001) increase in the number of vesicles contacting the plasma membrane (15.6% ± 0.75%, n = 74 synaptic profiles, for tom-1(ok285)) vs. 8.5% ± 0.3%, n = 250 synaptic profiles, for WT) (Figure 4A and 4B). In WT synapses, contacting vesicles were preferentially localized within ~150 nm of the presynaptic density (Figure 4C and 4D), whereas in tom-1(ok285) mutants the increase in membrane-contacting vesicles were distributed throughout the terminal (Figure 4C and 4D).

We next examined the ultrastructure of jaIs1052 (Figure 5). Since TOM-1A expression was restricted to the cholinergic motor neurons of tom-1(nu468) mutants in jaIs1052, we compared the contacting vesicle pool of cholinergic synapses in jaIs1052 versus tom-1(nu468) mutants (Figure 5A and 5B). Consistent with the reduced evoked response, cholinergic synapses in jaIs1052 had fewer contacting vesicles relative to tom-1(nu468) (7.9% ± 0.4%, n = 56 profiles, for jaIs1052 vs. 15.8% ± 0.6%, n = 49 profiles, for tom-1(nu468), p < 0.0001) (Figure 5B). As an internal control for the specificity of the TOM-1A rescue, we also examined the number of contacting vesicles in the GABAergic synapses of jaIs1052 worms (Figure 5D and 5E). The number of contacting vesicles was not significantly different (p > 0.5) between jaIs1052 GABA synapses (11.4% ± 1%, n = 20) and tom-1(nu468) (13.7% ± 0.8%, n = 19 profiles) (Figure 5E). Similarly, the distribution of vesicles in cholinergic synapses was reduced throughout the terminal in jaIs1052 cholinergic synapses (Figure 5C), but not in the GABAergic synapses of the same worms (Figure 5F). These data establish that vesicle contact is regulated by TOM-1 in both cholinergic and GABAergic synapses in C. elegans.
Increased Vesicle Priming in \textit{tom-1} Mutants

Since the formation of tomosyn SNARE complexes competes with the assembly of synaptobrevin-containing SNARE complexes, it has been proposed that tomosyn may inhibit synaptic vesicle priming. Therefore, the enhanced release and increased contacting vesicle pool in \textit{tom-1} mutants could reflect an increase in the size of the readily releasable pool. To directly measure the size of the readily releasable pool in \textit{tom-1} mutants, we recorded hyperosmotic responses at the neuromuscular junction. The hyperosmotic responses of both \textit{tom-1} alleles were significantly increased relative to the WT (18.4 ± 2.8 pC, \(n = 13\), for WT vs. 34.6 ± 5.1 pC, \(n = 6\), for \textit{tom-1}(n468), \(p = 0.0125\); and 28.8 ± 4.8 pC, \(n = 10\) for \textit{tom-1}(ok285), \(p = 0.049\)) (Figure 6), indicating that loss of TOM-1 results in an increased primed vesicle pool.

If the increased plasma membrane-contacting vesicle pool in \textit{tom-1} mutants were a reflection of enhanced priming, we would predict that the priming-defective mutant \textit{unc-13}(s69) would have fewer contacting vesicles. Morphometric analysis of \textit{unc-13} mutants revealed a profound reduction in membrane-contacting vesicles relative to the WT (3.5% ± 0.3%, \(n = 101\) synaptic profiles, for \textit{unc-13}(s69) vs. 8.5% ± 0.3%, \(n = 250\) synaptic profiles, for WT, \(p < 0.0001\)) (Figure 6A and 6B). This reduction is consistent with the proposed role of UNC-13 in promoting SNARE complex formation and thus, vesicle apposition with the plasma membrane.

Our analysis of \textit{tom-1} mutants suggests that priming is upregulated in the absence of TOM-1. Therefore, we next asked whether \textit{tom-1} mutants could suppress the synaptic defects associated with \textit{unc-13} mutants. In \textit{tom-1}(ok285) \textit{unc-13}(s69) double mutants the number of contacting vesicles relative to \textit{unc-13}(s69) increased to 5.9% ± 0.2% per profile (\(n = 124\) synaptic profiles, \(p < 0.0001\)) (Figure 6A and 6B). The distribution of contacting vesicles relative to the presynaptic density increased throughout the terminal in the \textit{tom-1}(ok285) \textit{unc-13}(s69) double mutants (Figure 6C). Coincident with this morphological rescue, \textit{tom-1}(ok285) \textit{unc-13}(s69) double mutants exhibited increased aldicarb sensitivity relative to \textit{unc-13}(s69) mutants, indicating that ACh release was partially restored (unpublished data). Recordings from the NMJ confirmed that both the evoked amplitude (3.3 ± 3.3 pA, \(n = 6\), for \textit{unc-13}(s69) vs. 752 ± 121 pA, \(n = 12\), for \textit{tom-1}(ok285) \textit{unc-13}(s69), \(p < 0.0006\)), and total charge integral (0.11 ± 0.05 pC, \(n = 6\), for \textit{unc-13}(s69) vs. 6 ± 1.2 pC, \(n = 11\), for \textit{tom-1}(ok285) \textit{unc-13}(s69), \(p < 0.0001\)) were increased in the double mutants with decay time constants similar to those of the WT.
The recovery of synaptic function in *tom-1(ok285) unc-13(s69)* mutants was accompanied by a corresponding increase in the size of the hyperosmotic response (5.3 ± 0.34 pC, n = 3, for *tom-1(ok285) unc-13(s69)*, vs. 1.17 ± 0.51 pC, n = 6, for *unc-13(s69)*, p = 0.001) to 30% of the WT (17.4 ± 3.3 pC, n = 11, for WT) (Figure 6E). Together, these data suggest that the priming defect of *unc-13* mutants can be partially ameliorated by removing TOM-1, further supporting the conclusion that TOM-1 functions to negatively regulate priming.

**Discussion**

**Summary**

We have examined the role of tomosyn in regulated synaptic transmission by analyzing two hypomorphic mutants of the *C. elegans* tomosyn homolog, *tom-1* [16]. Our results demonstrate that loss of TOM-1A and C isoforms enhances evoked release at the neuromuscular junction. In the absence of a complete *tom-1* null, the role of the remaining isoform, TOM-1B, remains to be elucidated. These results are consistent with a previously reported increase in sensitivity of *tom-1* mutants to the toxic effects of the acetylcholine esterase inhibitor, aldicarb, which is indicative of increased cholinergic transmission [16]. The enhanced neurotransmission observed electrophysiologically correlates with a redistribution of synaptic vesicles to the plasma membrane in *tom-1* mutant synapses. We further demonstrate that the ability of synaptic vesicles to contact the plasma membrane is regulated by the priming factor, UNC-13. When we generated *tom-1 unc-13* double mutants we observed partial suppression of the *unc-13* priming defect and concomitant restoration of the plasma membrane-contacting vesicle pool. Consistent with
previous studies of dense core vesicle fusion [6,10,12,13] and insulin release [14], these data suggest that tomosyn acts as a negative regulator of synaptic vesicle priming.

What Is the Molecular Mechanism by which Tomosyn Negatively Regulates Vesicle Priming?

Priming is thought to involve formation of SNARE complexes between synaptobrevin, syntaxin, and SNAP-25, which bring the vesicle membrane into close apposition with the plasma membrane [5,23]. Like vertebrate tomosyn [10], we demonstrate that the C-terminal of C. elegans TOM-1 also has the ability to form a complex with the SNARE domains of syntaxin and SNAP-25, suggesting this is a conserved tomosyn interaction. Vertebrate tomosyn competes with synaptobrevin for binding to syntaxin and SNAP-25 on the cytosolic surface of PC12 cell membrane sheets [10] as well as in in vitro biochemical studies [6]. By precluding vesicle-associated synaptobrevin from assembly into SNARE complexes, tomosyn is thus proposed to limit vesicle priming. This model is supported by several studies in which tomosyn overexpression has been shown to inhibit dense-core granule fusion [6,10,12] and neuronal exocytosis [15]. Furthermore, in

Figure 6. tomo-1(ok285) Suppresses the Synaptic Defects of unc-13(s69) Mutants

(A) Representative images of unc-13(s69) and tomo-1(ok285)unc13(s69). (B) The ratio of plasma membrane-contacting vesicles per profile for WT (black), unc-13(s69) (red), tomo-1(ok285)unc13(s69) (maroon), and tomo-1(ok285) (purple) scale bar = 200 nm. (C) Comparison of unc-13(s69) and tomo-1(ok285)unc13(s69) plasma membrane-contacting vesicle distribution relative to the PD in 30-nm bins. (D) Representative NMJ recordings demonstrate that the evoked response absent in unc-13(s69) mutants is partially restored in tomo-1(ok285)unc13(s69) double mutants. The average evoked charge integral of the tomo-1 unc-13 double mutants is graphed relative to WT and tomo-1(ok285) mutant responses. (E) Representative recordings of hyperosmotic responses demonstrate that the readily releasable pool of vesicles is increased in tomo-1(ok285) relative to WT. The hyperosmotic response absent in unc-13(s69) mutants is also partially restored in tomo-1(ok285)unc13(s69) double mutants. The mean total charge integral for synaptic events in the first second of the hyperosmotic response is graphed. Data expressed as mean ± SEM.

DOI: 10.1371/journal.pbio.0040261.g006
chromaffin cells, tomosyn overexpression specifically inhibits
the fast exocytotic burst corresponding to the fusion-
competent primed granule pool [12]. In contrast to over-
expression data, RNAi–mediated knockdown of tomosyn has
produced mixed results. In cultured neurons tomosyn RNAi
causes a reduction in synaptic vesicle release [15], where as
RNAi in beta cells results in enhanced exocytosis [14]. This
former study follows a previous report indicating that
tomosyn RNAi profoundly inhibits directed neurite out-
growth, which could affect synaptogenesis under these
culture conditions, resulting in fewer functional synapses
[19]. In the context of an intact animal examined here, loss of
tomosyn function does not appear to be deleterious for
neurite outgrowth and synaptogenesis. Therefore, the en-
hanced neurotransmission we observe in C. elegans tom-1
mutants is more consistent with RNAi in beta cells and appears
to be attributable to functional changes in release properties
rather than aberrant neuronal cytoarchitecture.
Specifically, phenotypic analysis of C. elegans tom-1 mutants
supports the hypothesis that tomosyn acts as a negative
regulator of synaptic vesicle priming in vivo.

In contrast to the C. elegans tom-1 mutant ultrastructural
phenotype, tomosyn overexpression in chromaffin cells was
not associated with any changes in granule distribution based
on conventional EM [12]. In TOM-1A–overexpressing synap-
ses, we also observe near-normal numbers of contacting
vesicles. Therefore, there is no conflict between the ultra-
structural data reported for tomosyn overexpression and that
of the present study. However, it remains to be seen whether
loss of tomosyn in chromaffin cells is associated with increased
granule association with the plasma membrane.

Why Does tom-1(ok285) Suppress the Synaptic Defect of
unc-13(s69)?

UNC-13 is a member of a conserved family of synaptic
proteins implicated in vesicle priming [24–26]. The mamma-
lian homolog Munc13–1 is thought to interact with the N-
terminus of syntaxin [27]. Syntaxin can adopt a closed state in
solution that occludes the syntaxin SNARE domain (the H3
domain) required for SNARE complex formation [28], UNC-
13 binding to the N-terminus of syntaxin has been proposed
to stabilize the syntaxin open configuration, increasing
accessibility of the H3 domain [27]. Partial suppression of
the C. elegans unc-13 priming defect by overexpression of a
constitutively open form of syntaxin supports this model [29].
Furthermore, mutations that disrupt UNC-13/syntaxin inter-
actions in vitro have been shown to reduce release in both C.
elegans and chromaffin cells [30,31]. However, a Munc13
domain capable of partially rescuing priming in Munc13 KO
mice, fails to bind syntaxin [32], suggesting alternative
molecular mechanisms could also account for the priming
function of Munc13. Here we show that tom-1(ok285) can also
partially suppress the unc-13(s69) mutant phenotype. We
postulate that in unc-13 mutants, any transitions of syntaxin
to the open state are prevented from forming functional
SNARE complexes through the binding of tomosyn. It is also
possible that the presence of UNC-13 normally excludes
tomosyn from interacting with syntaxin and SNAP-25. In
either case, we propose that in tom-1 unc-13 double mutants,
syntaxin has an increased probability of assembling into
SNARE complexes because tomosyn no longer precludes
synaptobrevin binding to the plasma membrane SNAREs.

Why Is the Postsynaptic Response Prolonged in C. elegans
tom-1 Mutants?

Many of the contacting synaptic vesicles in tom-1(ok285)
mutants are found distal to the presynaptic specialization.
If, as at vertebrate NMJs [33], calcium channels are localized
to the presynaptic specialization in C. elegans, calcium-
triggered fusion of distal vesicles may be delayed relative to
the proximal vesicles, resulting in a prolonged muscle
response. It is also possible that the increased distance
between distal neurotransmitter release sites and postsy-
naptic receptors prolongs the postsynaptic response in tom-1
mutant muscles.

How Might Tomosyn Restrict the Membrane Localization
of Synaptic Vesicles?

One mechanism by which tomosyn may restrict synaptic
vesicle fusion has recently emerged from the study of
neurite outgrowth following tomosyn RNAi [19]. In cultured
hippocampal neurons, knockdown of tomosyn increases
neurite sprouting and branching while reducing neurite
extension. This regulation is proposed to involve a high-
affinity interaction between tomosyn and phosphorylated
syntaxin (Ser14), which colocalize to the palms of growth
cones [19]. The phosphorylation of syntaxin (Ser14p) by
serine/threonine kinase (ROCK) leads to a five-fold increase
in binding of tomosyn relative to synaptobrevin. Synapto-
brevin-associated vesicles are thus predicted to be excluded
from priming in growth cone palms, fusing instead at the
leading edge of the growth cone, promoting neurite
extension. Whether a similar mechanism functions to
spatially restrict synaptic vesicle fusion to the active zone
region in synaptic terminals remains to be investigated.
However, syntaxin Ser14p is found throughout the rat
cortex and appears to be excluded from regions rich in
synaptic vesicles [34]. Therefore, it seems entirely possible
that tomosyn may act to restrict exocytosis at synaptic
terminals. Since syntaxin can be phosphorylated at Ser14 by
both ROCK [19] and casein kinase II [34], this could also
present a possible mechanism to regulate synaptic efficacy
by affecting the level of tomosyn-mediated inhibition of
vesicle priming.

The interaction between tomosyn and syntaxin is also
regulated by protein kinase A–dependent phosphorylation of
tomosyn in the variable linker between the WD40 domain
and the C-terminal SNARE homology domain. Specifically,
phosphorylation of tomosyn reduces the binding affinity of
the tomosyn–syntaxin interaction [15]. Furthermore, protein
kinase A–dependent synaptic facilitation appears to act in
part through the phosphorylation of tomosyn, suggesting
again that the interaction between tomosyn and syntaxin
negatively regulates neurotransmitter release.

In summary, tomosyn interacts with syntaxin and SNAP-25
to form tomosyn SNARE complexes predicted to be non-
fusogenic, which compete with SNARE complex formation
and thus inhibit vesicle priming. Tomosyn perturbation
analysis in C. elegans provides the first in vivo evidence in
support of this model. Tomosyn mutants not only exhibit
enhanced release but also suppress the priming defects of unc-
13 mutants. Future studies can now address whether the
regulation of tomosyn function by kinases provide mecha-
nisms for synaptic plasticity.
Materials and Methods

Genetics. Nematodes were maintained on agar plates seeded with OP50 bacteria. Strains used were N2 Bristol, VC223 tom-1(ok285), BC168 unc-13(69), SY1020 tom-1(ok285)unc-13(69), SY1016 tom-1(ok285)unc-13(69), and SY1181 tom-1(ok285); jals1052 (integrated Pun17:tom-1A).

In vitro biochemistry. Purification of recombinant proteins SNAREs lacking TM domains. Vectors for C. elegans SNAP-25 and syntaxin were constructed as follows: the entire SNAP-25 coding region was inserted into pPD9 (35), the syntaxin cytoplasmic domain (aa 1–266) was cloned into pGEX-2T, and the SNC-1 cytoplasmic domain (aa 1–88) was cloned into pRSETC. The TOM-1 SNAP-25 motif/2T7-codon coding region (TOM-1CT; aa 1,137–1,211) was subcloned into pRSETC and purified as an N-terminally his-tagged protein. Rat SNAP-25, HerS6, and His6 were expressed in [5] and purified by PhosphoImmun technologies (Washington University School of Medicine, Saint Louis, Missouri, United States). Proteins were purified using Ni-NTA resin or glutathione agarose, followed in some cases by further purification on a Mono S column using fast protein liquid chromatography.

Methodology. Fusion proteins were batch-purified on Ni-NTA-agarose according to the manufacturer’s protocols (Qiagen, Valencia, California, United States) under native conditions with modified buffers. The lysis buffer consisted of 20 mM HEPES (pH 7.4), 500 mM KCl, 0.1 mM PMSE, 0.1% BME, 5% glycerol, and 0.5 mM imidazole. The wash buffer consisted of 20 mM HEPES (pH 7.9), 200 mM KCl, 0.1 mM PMSE, 0.1% BME, 0.5% glycerol, 0.5% Triton X-100, and 1–300 mM imidazole. The dialysis buffer consisted of 10 mM HEPES (pH 7.9), 140 mM KCl, and 0.5% Triton X-100. The syntaxin-GST fusion protein and GST alone were batch-purified with glutathione agarose under native conditions [36]. The buffer differed from that used in the Ni-NTA-agarose purifications only as follows: the lysis buffer contained no imidazole and 100 mM EDTA; the wash and elution buffer contained no imidazole, 2 mM EDTA, and 15 mM reduced glutathione. The purity of the fractions was verified by SDS-PAGE, and the concentration was determined by the Bradford assay.

Tom-1 cDNA expression assays. For in vitro complex formation, ~1.6 µM of each of C. elegans syntaxin–GST (or GST), rat SNAP-25, His6, and C. elegans His6-TOM-1CT (or -His6-SNB-1) were incubated from 2 h to overnight at 4 °C with rocking in D-buffer. The proteins were then washed four times with 400 µl D-buffer containing no imidazole and 100 mM EDTA; the wash and elution buffer contained no imidazole, 2 mM EDTA, and 15 mM reduced glutathione. The purity of the fractions was verified by SDS-PAGE, and the concentration was determined by the Bradford assay.

Behavioral assays. Thrashing assays in M solution were performed by quantifying the number of body bends/min. For head tap responses, worms were allowed a 1-min settling period after placement in the assay chamber with food, before recording responses to a head tap for 20 s. Brood size was quantified by plating ten L4 hermaphrodites of the indicated genotype onto individual plates and counting the number of young adult worms for each genotype. For each assay, worms were allowed to settle on plates for 24 h before counting. The cycle was defined as an interval between two expansions of the gut content after visible contraction of the body wall muscles.

Real-time PCR. C. elegans total RNA was isolated using a Trizol reagent as described by the manufacturer (Invitrogen, Carlsbad, California, United States). Total RNA (0.5 µg) was used for reverse transcription using the SuperScript III First Strand Synthesis Kit (Invitrogen). Absence of genomic DNA was confirmed by PCR with primers designed to amplify the GFP coding region. GFP is a high copy coinjection marker, and we did not detect any genomic DNA contamination. Quantification of the relative amount of isoform A, based on a calibrator (dynamin). After RT-PCR, the products were subjected to agarose gel electrophoresis and sequencing to confirm the specificity of amplified products.

Immunohistochemistry. Immunohistochemistry was performed using a modified method of the whole worm fixation [57]. Briefly, the worms were frozen on dry ice and then in 3% glutaraldehyde, which were then split longitudinally, frozen animals were stored in 4% glutaraldehyde in PBS. Following fixation, animals were spun down at 1,600 rpm. Antibodies against UNC-29 (kindly provided by Dr. K. G. Miller) and UNC-17 (kindly provided by Dr. J. B. Rand) were used at a final dilution of 1:200 in PBS and 0.5% Triton X-100 with 0.3% BSA. Anti-rabbit and anti-mouse TRITC-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, United States) were used at 1:500 dilution for 4 h at 4 °C. Images were obtained with a 60x objective using an Olympus Optical (Tokyo, Japan) FV-500 laser-scanning confocal microscope. Puncta size and quantity were quantified using ImageJ software. A synapse was defined as a set of serial sections in the ventral nerve cord region upstream of the vulva using ImageJ.

Generation of TOM-1A rescuing lines. jals1052 strains expressing an integrated array of TOM-1A in tom-1(nu468) mutants were generated by irradiating animals expressing an extrachromosomal array of TOM-1A cDNA and a Phsy2-Xgfp coinjection marker under the cholinergic neuronal unc-17 promoter (kindly provided by Dr. J. Kaplan) using a Cs source (total dose of 1,800 rad).

Electrophysiology. Electrophysiological methods were as previously described [25]. Briefly, animals were immobilized with viscous sucrose gel, and a lateral cuticle incision was made exposing the ventral medial body wall muscles. Muscle recordings were made in the whole-cell voltage-clamp configuration (holding potential, −60 mV) using an EPC-10 patch-clamp amplifier and digitized at 2.9 kHz. The extracellular solution consisted of 150 mM NaCl, 5 mM KCl, 5 mM glucose, 5 mM K2HPO4, 10 mM HEPES (pH 7.3, −340 mOsm). The patch pipette was filled with 120 mM KCl, 20 mM KOH, 4 mM MgCl2, 5 mM (N-Tris[Hydroxymethyl] methyl-2-aminoethane-sulfonic acid), 0.25 mM CaCl2, 4 mM NaATP, 36 mM sucrose, and 5 mM EGTA (pH 7.2, −315 mOsm). Hyperosmolar solutions were recorded using an 800 mOsm extracellular solution achieved through addition of sucrose. Data were acquired using Pulse software (HEKA, Southborough, Massachusetts, United States) run on a Dell computer. Subsequent analysis and graphing was performed using Pulsefit (HEKA). Mini analysis (Synaptosoft Inc., Decatur, Georgia, United States) and Igor Pro (Wavemetrics, Lake Oswego, Oregon, United States).

EM. N2, tom-1(ok285), tom-1(nu468), tom-1(ok285)unc-13(s69), unc-13(s69), and TOM-1A rescue young-adult hermaphrodites were prepared for high-pressure freezing as previously described [22]. Briefly, ten to 15 animals were loaded in a specimen chamber filled with E. coli and immobilized by high-pressure freezing at −180 °C under high pressure in a Bal-Tec HPM010 and moved to liquid nitrogen.

Freeze substitution was performed in a Reichert AFS machine (Leica, Wetzlar, Germany) on a Reichert-Jung Ultracut E (Leica, Melrose Park, Illinois, United States) using 3% tannic acid (0.1%) and 0.5% glutaraldehyde fixative introduced over 4 d followed by 2% osmium. Fixed animals were then washed and embedded in Araldite 502 over a 48-h period at 60 °C.

Serial sections were cut at a thickness of 40–50 nm, collected on formvar-covered carbon coated copper grids (EMS, FC2010-Co), and counterstained in 2% or 2.5% aqueous uranyl acetate for 4 min, followed by Reynolds lead citrate for 2 min. Images were obtained on a JEM-1220 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV. Micrographs were collected using a Gatan digital camera (Pleasanton, California, United States).

Morphometric analysis. WT, tom-1(ok285), tom-1(nu468), tom-1(ok285)unc-13(s69), unc-13(s69), and jals1052 TOM-1A rescued animals were performed from ventral nerve cord serial sections. The analysis was performed blind. Images were quantified using NIH Image software. A synaptic profile was defined as a set of serial sections containing a presynaptic specialization and two flanking sections from both sides without presynaptic specialization. Several morphometric measurements were obtained: the number of profiles per profile, the distance from each vesicle membrane perpendicular to the plasma membrane, and the distance to the proximal edge of the synapse.

Supporting Information

Figure S1. Coomassie Blue–Stained PAGE Gel of Mixed-Species SNARE Complex Assembly Assays

Complex assays were performed using C. elegans His6-tagged SNAREs.
and rat His6-tagged SNAREs. Purified syntaxin, SNAP-25, and synaptobrevin were mixed, incubated to allow complex formation, and separated on a PAGE gel to visualize complexes. One aliquot of each sample was loaded without heating (rt), and the other was heated to 95 °C for 5 min (b) prior to loading. The position of complexes, syntaxin, SNAP-25, and C. elegans synaptobrevin (which migrates just above the dye front) are labeled on the gel. Unfortunately, vertebrate (v) synaptobrevin migrates in the dye front and is not identifiable on the gel. Various combinations of C. elegans (c) and rat protein (v) mixtures were tried. All reactions containing purified C. elegans SNAP-25 failed to form complexes efficiently, while all other combinations formed complexes. The positions of molecular weight markers are on the left.

Methods: C. elegans His6-tagged SNAREs and rat SNAP-25 were purified as described in the main text. Rat His6-tagged synaptobrevin and syntaxin were purified from Michael Crowder (Washington University School of Medicine) and purified as described in [29]. To form complexes, purified syntaxin, SNAP-25, and synaptobrevin were mixed, incubated overnight at 4 °C, mixed with SDS-containing sample buffer, and run on a 4%–12% PAGE gel.

Found at DOI: 10.1371/journal.pbio.0040261.sg002 (2.8 MB JPG).

Figure S2. C. elegans TOM-1Ct Does Not Form a Stable Binary Complex with Syntaxin

A Coomassie-stained gel shows GST pull-down assays performed to eliminate the possibility that TOM-1Ct forms a complex with syntaxin in the absence of SNAP-25. C. elegans GST syntaxin or GST alone was incubated with either rat SNAP-25 (vSNAP-25) or C. elegans tomosyn C-terminal domain (TOM-1Ct). Glutathione agarose beads were added to collect complexes. The supernatant was removed, and the beads were washed extensively. Aliquots of the supernatant (sup) were added to collect complexes. The supernatant was removed, and the beads were washed extensively. Aliquots of the supernatant (sup) and the protein eluted from the glutathione agarose (beads) were separated on SDS-PAGE and stained for protein with Coomassie Blue. Although syntaxin bound to SNAP-25 in the absence of TOM-1Ct or synaptobrevin, TOM-1Ct did not interact with syntaxin in the absence of SNAP-25. Molecular weights of markers are labeled on the left.

Found at DOI: 10.1371/journal.pbio.0040261.sg002 (529 KB JPG).

Figure S3. Enlarged Micrographs of Representative NMJ Used in the Study

(A) WT. (B) tom-1(ok285). (C) tom-1(nu468) cholinergic synapse. (D) Cholinergic synapse in jpk1052 (an integrated array of TOM-1A expressed in cholinergic neurons using the arc-2 promoter, in a toms-1(nu468) mutant background). (E) toms-1(nu468) GABAergic synapse. (F) GABAergic synapse in jpk1052 (an integrated array of TOM-1A expressed in cholinergic neurons using the arc-2 promoter, in a toms-1(nu468) mutant background). (G) unc-13(s69). (H) tom-1(ok285) unc-13(s69) double mutant. Arrows point to membrane-contacting synaptic vesicles.

Found at DOI: 10.1371/journal.pbio.0040261.sg003 (2.8 MB JPG).

Acknowledgments

We would like to thank Dr. Joshua Kaplan for supplying the TOM-1A extrachromosomal array integrated in this study; Dr. Aixa Alfonso for the C. elegans unc-17 mutant background; Dr. Jean-Louis Bessereau for UNC-29 antibodies; Dr. Phyllis Hanson and Dr. C. Michael Crowder for providing purified rat SNARE proteins; Drs. Yishi Jin for supplying the cholinergic GFP reporter strain (jpk134); Dr. Jean-Louis Bessereau (INSERM), Linda Juarez and Kristina Jarosius (RRC EM facility at UIC), and Jay Campbell with the LOCI lab at UW-Madison for technical assistance with the high-pressure freeze EM; and Dr. Robert Wykes and Dr. David Featherstone for constructive criticism of the manuscript.

Author contributions. EOG, AOB, AMH, MLN, RMW, and JER conceived and designed the experiments. EOG, AOB, AMH, MBG, JER, BDA, GH, and MLN performed the experiments. EOG, AOB, AMH, BDA, and MLN analyzed the data. MLN and RMW contributed reagents/materials/analysis tools. EOG, JER, and RMW wrote the paper.

Competing interests. The authors have declared that no competing interests exist.

Funding. Funding for this study was provided by National Institutes of Health (NIH) ROI NS41477 to JER and NIH R01 NS35353 to MLN and MDA3930 to BDA.
23. Chen YA, Scales SJ, Scheller RH (2001) Sequential SNARE assembly underlies priming and triggering of exocytosis. Neuron 30: 161–170.
24. Augustin I, Rosenmund C, Sudhof TC, Brose N (1999) Munc13–1 is essential for fusion competence of glutamatergic synaptic vesicles. Nature 400: 457–461.
25. Richmond JE, Davis WS, Jorgensen EM (1999) UNC-13 is required for synaptic vesicle fusion in C. elegans. Nat Neurosci 2: 959–961.
26. Aravamudan B, Fergestad T, Davis WS, Rodesch CK, Broadie K (1999) Drosophila UNC-13 is essential for synaptic transmission. Nat Neurosci 2: 965–971.
27. Betz A, Okamoto M, Benseler F, Brose N (1997) Direct interaction of the rat unc-13 homologue Munc13–1 with the N terminus of syntaxin. J Biol Chem 272: 2526–2528.
28. Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, et al. (1999) A conformational switch in syntaxin during exocytosis: role of munc18. EMBO J 18: 4372–4382.
29. Richmond JE, Weimer RM, Jorgensen EM (2001) An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. Nature 412: 338–341.
30. Madison JM, Nurrish S, Kaplan JM (2005) UNC-13 interaction with syntaxin is required for synaptic transmission. Curr Biol 15: 2236–2242.
31. Stevens DR, Wu ZX, Matti U, Junge HJ, Schirra C, et al. (2005) Identification of the minimal protein domain required for priming activity of Munc13–1. Curr Biol 15: 2243–2248.
32. Basu J, Shen N, Dulubova I, Lu J, Guan R, et al. (2005) A minimal domain responsible for Munc13 activity. Nat Struct Mol Biol 12: 1017–1018.
33. Robitaille R, Adler EM, Charlton MP (1990) Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses. Neuron 5: 773–779.
34. Foletti DL, Lin R, Finley MA, Scheller RH (2000) Phosphorylated syntaxin 1 is localized to discrete domains along a subset of axons. J Neurosci 20: 4535–4544.
35. Magittai M, Otto H, Jahn R (1999) A stable interaction between syntaxin 1a and synaptobrevin mediated by their transmembrane domains. FEBS Lett 446: 40–44.
36. Frangioni JV, Neel BG (1995) Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. Anal Biochem 210: 179–187.
37. Charlie NK, Schade MA, Thomure AM, Miller KG (2006) Presynaptic UNC-31 (CAPS) is required to activate the G (alpha)s pathway of the synaptic signaling network. Genetics, 172: 945–961.
38. Weiner RM. Preservation of C. elegans tissue via high-pressure freezing and freeze-substitution for ultrastructural analysis and immuno-cytochemistry. In: Strange K, editor. C. elegans: Methods and applications. Totowa, NJ: Humana Press. In press.
39. Nagele P, Mendel JB, Placzek WJ, Scott BA, D’Avignon DA, et al. (2005) Volatile anesthetics bind rat synaptic snare proteins. Anesthesiology 103: 768–778.