Transmembrane tethering of synaptotagmin to synaptic vesicles controls multiple modes of neurotransmitter release

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Synaptotagmin 1 (Syt1) is a synaptic vesicle integral membrane protein that regulates neurotransmitter release by activating fast synchronous fusion and suppressing slower asynchronous release. The cytoplasmic C2 domains of Syt1 interact with SNAREs and plasma membrane phospholipids in a Ca^{2+}-dependent manner and can substitute for full-length Syt1 in in vitro membrane fusion assays. To determine whether synaptic vesicle tethering of Syt1 is required for normal fusion in vivo, we performed a structure-function study with tethering mutants at the *Drosophila* larval neuromuscular junction. Transgenic animals expressing only the cytoplasmic C2 domains or full-length Syt1 tethered to the plasma membrane failed to restore synchronous synaptic vesicle fusion, and also failed to clamp spontaneous vesicle release. In addition, transgenic animals with shorter, but not those with longer, linker regions separating the C2 domains from the transmembrane segment abolished Syt1's ability to activate synchronous vesicle fusion. Similar defects were observed when C2 domain alignment was altered to C2B-C2A from the normal C2A-C2B orientation, leaving the tether itself intact. Although cytoplasmic and plasma membrane-tethered Syt1 variants could not restore synchronous release in *syt1* null mutants, they were very effective in promoting fusion through the slower asynchronous pathway. As such, the subcellular localization of Syt1 within synaptic terminals is important for the temporal dynamics that underlie synchronous and asynchronous neurotransmitter release.

synaptotagmin | *Drosophila* | synaptic vesicle | neurotransmitter release | exocytosis

Neurotransmitter release requires temporal and spatial coupling of action potential-triggered Ca^{2+} influx to synaptic vesicle fusion (1). The core fusion machine contains SNARE proteins found on the synaptic vesicle (v-SNAREs) and plasma membrane (t-SNAREs) that assemble into a four-helix bundle to bring the two bilayers into close apposition (2, 3). Besides SNAREs, Ca^{2+}-binding proteins act to trigger release through fast synchronous and slow asynchronous pathways. Synaptotagmin 1 (Syt1) is a synaptic vesicle protein that binds Ca^{2+} and triggers synchronous vesicle fusion (4–9). Syt1 contains an intravesicular N-terminal tail, a single transmembrane segment, and a ~60- residue linker that connects to two cytoplasmic Ca^{2+}-binding C2 domains (10–13).

Numerous Syt1 studies have focused on its cytoplasmic C2 domains, which interact with phospholipids and the SNARE complex in a Ca^{2+}-dependent manner and are proposed to be the essential domains that trigger fusion (12, 14–21). In contrast, the significance of other structural elements of Syt1 remains poorly understood. Syt1 is predicted to facilitate synaptic vesicle fusion through a *trans* interaction with plasma membrane lipids (22–27). Tethering of Syt1 to synaptic vesicles through its transmembrane domain has been postulated to position the protein to properly target lipids and SNAREs, or to be required to generate force for pulling the membranes together. Although anchoring through the transmembrane tether is unlikely to generate the intramembrane proximity

required for the final steps in fusion owing to the distance involved, binding of individual C2 domains simultaneously to both membranes might, because such binding can aggregate lipid bilayers in vitro (27–29).

Despite these models, however, the role of vesicular tethering of Syt1 in vivo remains unclear. Injection of a cytoplasmic domain of rat Syt1 into crayfish motor axons facilitates exocytosis (30), implying that the cytoplasmic region alone may act as a fusion trigger. In contrast, in vitro studies indicate that the linker domain that connects the transmembrane region to the C2 domains may regulate docking, fusion pore opening, Syt1 multimerization, and intramolecular C2 domain interactions (31–34). The requirement of C2 domain order (C2A, then C2B) has been suggested to be dispensable for synaptic vesicle endocytosis in vitro (35), but the functional consequences of altered C2 domain order on Syt1's role in triggering exocytosis in vivo remain unclear.

Here we assayed the requirements of these Syt1 regions for neurotransmitter release in vivo. We generated transgenic animals expressing modified Syt1 proteins in the *synaptotagmin 1* null mutant background and examined their function at the *Drosophila* larval neuromuscular junction (NMJ), a well-established model glutamatergic synapse. Our results indicate that synaptic vesicle tethering, optimal linker length, and specific C2 domain alignment are important for Syt1 to regulate vesicle fusion. In addition, synaptic vesicle-tethered and cytoplasmic Syt1 proteins differentially regulate synchronous vs. asynchronous release kinetics, indicating that synaptic vesicle localization of Syt1 is critical for regulating neurotransmitter release.

Significance

Synaptotagmin 1 (Syt1) is widely considered to act as the fast Ca^{2+} sensor for synchronous synaptic vesicle fusion through its tandem Ca^{2+}-binding C2 domains. Here we demonstrate that Syt1's C2 domains activate rapid synchronous fusion only if they are in the proper orientation and specifically tethered to the synaptic vesicle with an appropriate linker distance. Although expression of the cytoplasmic C2 domains of Syt1 alone did not support fast synchronous release, it did enhance the asynchronous component of exocytosis. These findings demonstrate that synaptic vesicle tethering of Syt1 positions the protein to allow its C2 domains to regulate the kinetics of vesicle fusion.

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Results

Tethering of Synaptotagmin 1 to Synaptic Vesicles Differentially Alters Synchronous vs. Asynchronous Fusion. In vitro studies on Syt1 have focused largely on its cytoplasmic domain, which can facilitate Ca^{2+}-dependent fusion similar to the full-length counterpart in liposome fusion assays (22, 36–39). Here we generated transgenic *Drosophila* Syt1 constructs with no transmembrane tethering (cytoplasmic C2A-C2B), varying linker distance (no linker and 2× linker C2A-C2B), or altered C2 domain order [C2B-C2A (flipped) instead of C2A-C2B] (Fig. 1A). We also generated a cytoplasmic version with altered Ca^{2+} binding (cytoplasmic C2A*-C2B*) by mutating two of the five key aspartate residues in both C2 domains (D282N, D284N, D416N, and D418N; Fig. 1A, white circles). To eliminate the effects of genomic position on transgenic expression, we used site-specific transformation via the ΦC31 integrase system (40).

To determine whether these manipulations affected stability or synaptic targeting of Syt1 transgenic constructs, we performed Western blot and immunostaining analyses in *syt1* null mutants ($syt1^{AD4}/syt1^{N13}$, referred to hereinafter as $syt1^{-/-}$) expressing the transgenes driven by the pan-neuronal driver, $elav^{C155}$-GAL4 (C155). Lower levels of protein expression were observed from brain lysates expressing the shortened linker (no linker) and altered C2 domain order (flipped) versions, whereas the cytoplasmic Syt1 proteins were expressed at normal levels (Fig. 1B). All Syt1 variants, including the no linker and flipped versions, targeted normally to *Drosophila* larval NMJs (Fig. 1C), suggesting that these structural alterations do not perturb synaptic Syt1 localization.

We next assayed the functional effects of these transgenic proteins by measuring excitatory junction potentials (EJPs) following nerve stimulation to determine if they rescued the Ca^{2+}-dependent synchronous fusion that is missing in the absence of endogenous Syt1. Transgenic animals expressing only the cytoplasmic domain of Syt1 (cytoplasmic C2A-C2B) demonstrated dramatically altered kinetics of evoked responses compared with $syt1^{-/-}$ animals rescued with full-length WT Syt1 (C2A-C2B) (Fig. 2A). We quantified the synchronous and asynchronous components by assaying voltage changes in 100-ms intervals for 100 ms of prestimulation and 500 ms of poststimulation (Fig. 2B, Inset). Unlike WT, cytoplasmic Syt1 dramatically facilitated the asynchronous component of synaptic responses, at the expense of synchronous release occurring within the first 100-ms window. (Statistical analyses of datasets are reported in Table S1.)

Such enhanced asynchronous release may reflect a defect in concentrating Syt1 at release sites, which could compromise the kinetics of rapid Ca^{2+} binding and triggering of fusion. If so, cytoplasmic Syt1 would still bind Ca^{2+}, but would take longer to engage membranes and SNARE complexes, given that it was not prepositioned on synaptic vesicles. However, the shift in release kinetics observed with cytoplasmic Syt1 did not depend on its ability to bind Ca^{2+}, because $syt1^{-/-}$ null mutants rescued with a Ca^{2+}-binding–defective cytoplasmic construct (cytoplasmic C2A*-C2B*) showed a similar shift as seen on enhanced asynchronous release (Fig. 2). In contrast, a full-length Ca^{2+}-binding–deficient Syt1 (C2A*-C2B*) that was normally tethered to synaptic vesicles did
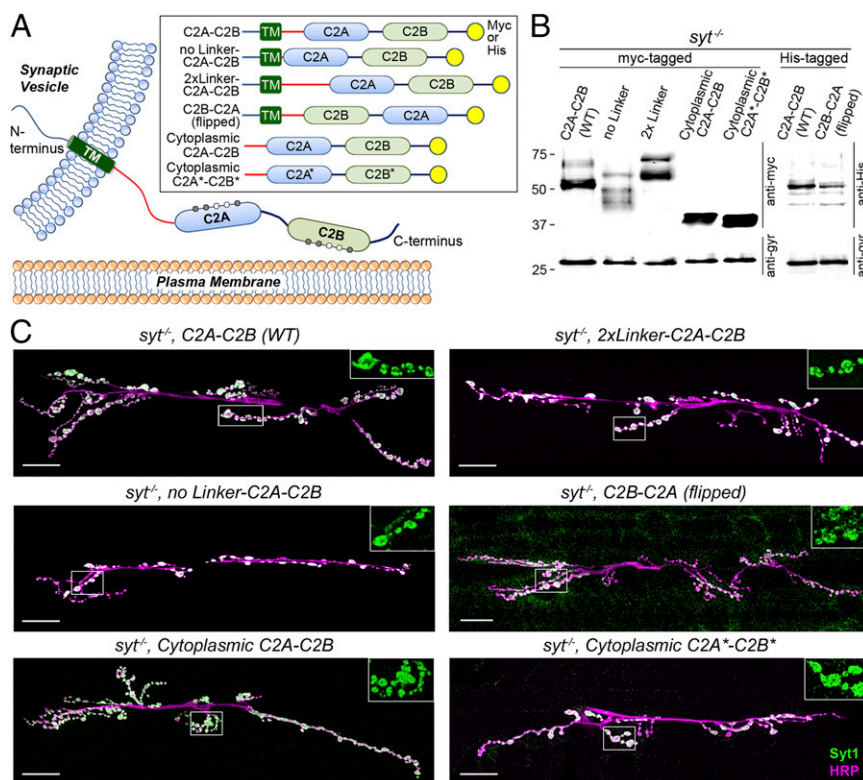

Fig. 1. Generation of transgenic synaptotagmin 1 constructs and their targeting to synapses. (A) Schematic of Syt1 at the vesicle–membrane interface. Syt1 transgenic proteins tagged with Myc or His (yellow circles) were generated for WT (C2A-C2B) and other variants containing virtually no or a double-length (2×) linker segment, a flipped C2B-C2A orientation, and cytoplasmic C2A-C2B alone or with mutated Ca^{2+}-binding residues (cytoplasmic C2A*-C2B*). The five aspartate residues critical for Ca^{2+} binding in each C2 domain are indicated as small circles, two of which are mutated in the C2A*-C2B* construct (white circles). (B) Western blot analysis for expression of transgenic Syt1 proteins in adult *Drosophila* brain lysates. The transgenic proteins were detected with anti-myc or anti-His antibodies in the *syt1* null mutant background ($syt^{-/-}$). Antisera to synaptogyrin (gyr) were used for a loading control. Several of the transgenic proteins have some smaller degradation products that are weakly visible, and we cannot rule out unexpected effects from such products. (C) Distribution of transgenic Syt1 proteins at larval NMJs visualized with anti-Syt1 (green) antisera in the $syt^{-/-}$ background. Anti-HRP antibody (magenta) was used to counterstain the synaptic arbor. (Insets) Magnified images of Syt1 immunoreactivity for the boxed regions. (Scale bar: 20 μm.)
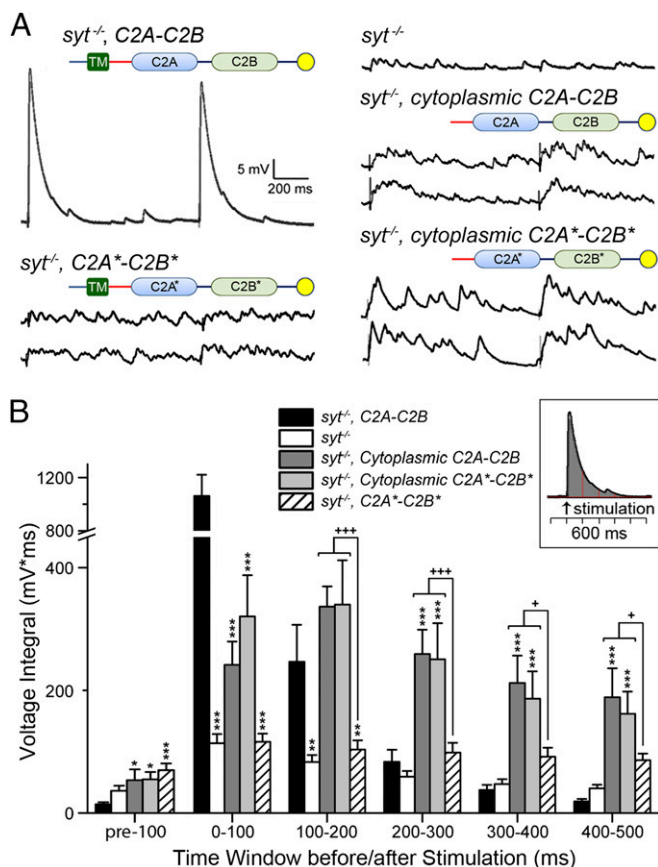
Fig. 2. Enhancement of asynchronous release by cytoplasmic synaptotagmin 1. (*A*) Representative traces of consecutive EJPs recorded in HL3.1 saline with 1.0 mM extracellular [Ca^{2+}] shown for *syt1* null mutants (*syt$^{-/-}$*) rescued with the indicated transgenic constructs. (Scale bars: 5 mV and 200 ms.). (*B*) Voltage integral (mV × ms) values from EJP responses plotted for the indicated time bins pre- and poststimulation for the specified genotypes. (*Inset*) Calculation of voltage integral in 100-ms bins (red vertical lines). Mean ± SEM are indicated. The numbers of larva examined were as follows: *syt$^{-/-}$*, C2A-C2B (WT), 10; *syt$^{-/-}$*, 12; *syt$^{-/-}$*, cytoplasmic C2A-C2B, 7; *syt$^{-/-}$*, cytoplasmic C2A*-C2B*, 4; *syt$^{-/-}$*, C2A*-C2B* (full-length), 10. **$P < 0.01$; ***$P < 0.001$, one-way ANOVA for WT vs. genotypes indicated at a 100-ms interval. $^+P < 0.05$; $^{+++}P < 0.001$, Fisher's least significant difference (LSD) multiple-comparison test for each pair indicated.

not enhance asynchronous release (Fig. 2*B*). These data indicate that relocation of Syt1 to the cytoplasm from synaptic vesicles, regardless of its Ca^{2+}-binding ability, shifts synaptic vesicle release from a synchronous mode to an asynchronous mode. The increase in asynchronous release was present only in the absence of endogenous Syt1, given that overexpression of these cytoplasmic constructs in the WT background did not yield changes in the temporal profiles of synaptic responses (Fig. S1).

It should be noted that substantial increases in the rate of spontaneous vesicle release, or miniature EJPs (mEJPs), also could contribute to voltage changes measured in the poststimulation window. To evaluate this contribution, we analyzed voltage changes at the 100-ms prestimulation window in *syt1$^{-/-}$* null mutants rescued with transgenic constructs. However, the slightly elevated prestimulation voltage integral in cytoplasmic Syt1-expressing animals fell far short of explaining the robust increase in stimulation-induced asynchronous release (Fig. 2*B*). In the absence of nerve stimulation, we detected increases in mEJP frequency, in addition to asynchronous release, in animals expressing cytoplasmic Syt1 [9.16 ± 1.62 Hz for cytoplasmic C2A-C2B ($n = 7$) vs. 2.74 ± 0.29 Hz for full-length C2A-C2B ($n = 15$); $P < 0.001$] (Fig. S2).

However, the full-length C2A*-C2B* rescue construct promoted spontaneous release to far greater levels than its cytoplasmic counterpart [17.28 ± 1.54 Hz for C2A*-C2B* ($n = 9$) vs. 4.81 ± 0.96 Hz for cytoplasmic C2A*-C2B* ($n = 7$); $P < 0.001$] (Fig. S2), but did not alter asynchronous release (Fig. 2*B*). Thus, enhanced spontaneous release cannot explain the increased asynchronous fusion observed with cytoplasmic Syt1. These results indicate that tethering of Syt1 to synaptic vesicles is indispensable for its function to selectively facilitate fast synchronous vesicle fusion.

Targeting of Synaptotagmin 1 to the Plasma Membrane Fails to Support Synchronous Vesicle Release. Currents models suggest that Syt1 may bring the vesicle and plasma membranes in close proximity through its attachment to synaptic vesicles and Ca^{2+}-dependent penetration into the plasma membrane via the C2 domains (27, 37, 39). However, in vitro studies indicate that synaptic transmission can be supported by a plasma membrane-tethered Syt1 construct (35, 39). Thus, we investigated whether alternative targeting of Syt1 to the plasma membrane could functionally replace its endogenous tethering to synaptic vesicles in vivo. We generated a transgenic construct in which the N-terminal region of Syt1, including the transmembrane domain, was replaced with a myristoylation motif (myr-C2A-C2B or myr-Syt1 hereinafter), a lipid anchor that has been successfully used in *Drosophila* to target other proteins to the synaptic plasma membrane in vivo (41) (Fig. 3*A*). Endogenous Syt1 displays a characteristic halo-like distribution pattern at synapses that corresponds to synaptic vesicles distributed throughout the bouton, including the interior of the terminal (Fig. 3*B*, *Upper*, arrowheads). In contrast, myr-Syt1 was localized at the periphery of synaptic terminals (Fig. 3*B*, *Lower*, arrows), with increased colocalization with syntaxin (Syx), a plasma membrane t-SNARE protein [Fig. 3*C*; coefficient for colocalization of Syt1 relative to Syx (*Left*): 0.51 ± 0.03 for C2A-C2B vs. 0.66 ± 0.02 for myr-Syt1, $P < 0.01$; coefficient for overall colocalization between Syt1 and Syx (*Right*): 0.27 ± 0.03 for C2A-C2B vs. 0.36 ± 0.02 for myr-Syt1, $P < 0.05$].

We next assayed the ability of myr-Syt1 to restore synaptic responses in *syt1$^{-/-}$* mutants. In contrast to WT, myr-Syt1 failed to restore synchronous release and clamp spontaneous fusion (Fig. 3*C*, Fig. S2, and Table S2). In addition, myr-Syt1 resulted in a significant increase in asynchronous vesicle release, a pattern indistinguishable from that observed with cytoplasmic Syt1 (Fig. 3*D*, green column). These data indicate that tethering of Syt1 to synaptic vesicles, but not to the opposing plasma membrane, is required to properly activate synchronous release and clamp spontaneous fusion.

Synaptotagmin 1 Linker Domain Length and C2 Domain Arrangement Regulate Synchronous Fusion. Given that Syt1 requires tethering to synaptic vesicles to selectively promote synchronous neurotransmitter release, we assayed how the spacing of the C2 domains from the vesicle membrane, as well as C2 domain order, would alter synaptic transmission. Syt1 proteins expressing an extended double-length linker domain (2× linker) rescued synaptic transmission defects in *syt1$^{-/-}$* mutants comparable to their WT counterpart (Fig. 4 *A* and *B*). In contrast to the extend linker, *syt1$^{-/-}$* mutants rescued with virtually no linker failed to restore normal evoked responses, with responses indistinguishable from those of *syt1$^{-/-}$* (Fig. 4 *A* and *B*). We did not detect any significant differences in asynchronous responses occurring between 200 and 500 ms poststimulation among *syt1$^{-/-}$* mutants rescued with WT, 2× linker, and no linker Syt1 constructs (Table S3). The no linker version exhibited mildly enhanced spontaneous release [6.15 ± 0.73 Hz ($n = 10$); Fig. S2] that resulted in a slight elevation in the voltage integral that was similar in the prestimulation and poststimulation 400- to 500-ms windows (Fig. 4*B* and Table S3). These results indicate that the linker domain has a minimal length requirement to facilitate synchronous release and to clamp spontaneous fusion.
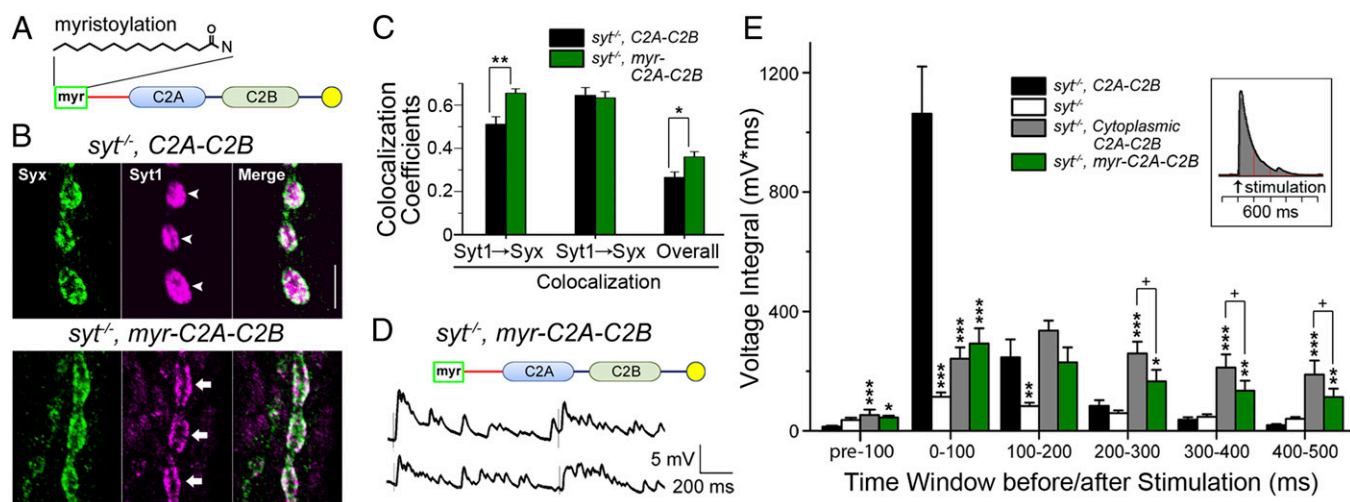
Fig. 3. Failure to restore synchronous release by plasma membrane-targeted synaptotagmin 1. (*A*) Schematic of myr-Syt1 showing the intravesicular tail and transmembrane segment replaced with a myristoylation motif to target the protein to the plasma membrane. (*B*) Distribution of WT (C2A-C2B) or myr-Syt1 expressed in the *syt1* null mutant background (*syt*$^{-/-}$) revealed by immunoreactivity against Syt1 (magenta) and compared with the plasma membrane t-SNARE syntaxin (Syx) (green). Note that myristoylation of Syt1 increases targeting of the protein from central areas of the boutons (*Upper*, arrowheads) to the plasma membrane domain (*Lower*, arrows) defined by Syx immunoreactivity. (*C*) The fraction of colocalization between Syt1 and Syx calculated from double-labeled confocal images, using ImageJ with the Coloc2 plugin (*SI Materials and Methods*). Manders and Pearson coefficients are indicated for relative [Syt1 to Syx (*Left*) and Syx to Syt1 (*Center*)] and overall colocalization (*Right*) between the two signals, respectively. The numbers of larva and regions of interest (ROIs) examined were as follows: *syt*$^{-/-}$, *C2A-C2B* (WT), 5 (13); *syt*$^{-/-}$, *myr-C2A-C2B*, 6 (12). *$P < 0.05$; **$P < 0.01$, Student two-sample *t* test for WT vs. *myr-C2A-C2B*. (*D*) Representative traces of EJP responses shown for *syt1*$^{-/-}$ mutants rescued with myristoylated Syt1. (Scale bar: 5 mV and 200 ms.) (*E*) Voltage integral (mV × ms) values plotted for the indicated time bins pre- and poststimulation (green column for myr-Syt1). Data from the *syt1*$^{-/-}$ (white column) and rescue with WT (black column) and cytoplasmic Syt1 proteins (gray column) from Fig. 2 are shown for comparison. Mean ± SEM are indicated. The numbers of larva examined were as follows: *syt*$^{-/-}$, *C2A-C2B* (WT), 10; *syt*$^{-/-}$, 12; *syt*$^{-/-}$, *cytoplasmic C2A-C2B*, 7; *syt*$^{-/-}$, *myr-C2A-C2B*, 9. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, one-way ANOVA for WT vs. genotypes indicated at a 100-ms interval. $^+P < 0.05$, Fisher's LSD multiple-comparison test for each pair indicated.

Although the C2A and C2B domains have several distinct effector interactions, it is unclear whether the specific alignment of C2A preceding C2B is a core feature of Syt1. To address this question, we analyzed synchronous release in *syt1*$^{-/-}$ mutants rescued with a flipped Syt1 C2 domain order (C2B-C2A). Unlike the ability of a C2B-C2A flipped construct to rescue endocytosis (35), C2B-C2A failed to restore the synchronous component of vesicle release in vivo in *syt1*$^{-/-}$ mutants (Fig. 4 *A* and *B*). The flipped Syt1 rescue also displayed enhanced spontaneous release [5.34 ± 0.74 Hz (*n* = 5); Fig. S2], resulting in a mildly elevated voltage integral at 400–500 ms poststimulation and, to a lesser extent, at 100 ms prestimulation (Fig. 4*B* and Table S3). These results indicate that the specific C2A-C2B orientation of the cytoplasmic C2 domains is required for synchronous neurotransmitter release. Given the presence of the intact linker domain in this line, the data also suggest that the linker domain alone is insufficient to restore the normal kinetics of release.

Discussion

The Ca^{2+}-binding C2 domains of Syt1 have been intensively studied for their role in driving synchronous synaptic vesicle fusion. Here we analyzed whether other regions of Syt1 also participate in regulating release. Our findings demonstrate that transmembrane tethering to synaptic vesicles and maintenance of the linker length and C2 domain orientation are critical for Syt1 to regulate neurotransmission. In addition, cytoplasmic Syt1 enhanced asynchronous release even in the presence of Ca^{2+}-binding mutations in both C2 domains, indicating that vesicular tethering of Syt1 is important for whether fusion occurs through a synchronous pathway or an asynchronous pathway. Taken together, these data demonstrate that synaptic vesicle tethering and linker domain length function to allow the C2A-C2B domains of Syt1 to regulate multiple modes of neurotransmitter release.

One goal of this study was to compare the requirements of synaptic vesicle tethering of Syt1 in vivo to in vitro biochemistry and liposome fusion results that used the Syt1 cytoplasmic C2 domains (23–25, 36). Only a few previous in vivo studies have investigated whether Syt1 requires synaptic vesicle tethering, and have yielded conflicting results. Although injections of the cytoplasmic domain of rat Syt1 into crayfish motor axons appeared to enhance the synchronicity of release (30), a similar approach in *Aplysia* neurons found inhibitory effects of cytoplasmic Syt1 proteins (42). Using a genetic rescue approach, we found that the cytoplasmic domain of Syt1 could not support normal synaptic transmission in vivo. Cytoplasmic Syt1 failed to rescue characteristic defects of *syt1* null mutants, including disrupted synchronous evoked release and enhanced spontaneous fusion (Fig. 2 and Fig. S2). We hypothesize that synaptic vesicle tethering positions the C2 domains near plasma membrane lipids and the SNARE complex, given that interactions with these effectors have been suggested to mediate the activation of evoked release and suppression of spontaneous fusion.

In contrast to synchronous release, cytoplasmic Syt1 expression induced a novel effect on synaptic transmission that has not been reported in vitro: a dramatic enhancement of asynchronous release (Fig. 2). The *syt1*$^{-/-}$ mutants alone exhibited slightly elevated asynchronous release, indicating that Syt1 can suppress this slower fusion mode (7); however, cytoplasmic Syt1 triggered a long-lasting increase in asynchronous fusion far greater than that observed in *syt1*$^{-/-}$ (Fig. 2*B*). These data indicate that cytoplasmic Syt1 promotes asynchronous release, rather than simply failing to suppress the asynchronous pathway. We initially surmised that this enhanced release might be due to Ca^{2+}-bound Syt1 taking longer to engage membrane lipids or SNARE complexes, given that it was not positioned normally at the site of fusion through membrane tethering. This hypothesis was not supported by the findings that Ca^{2+}-binding–defective cytoplasmic
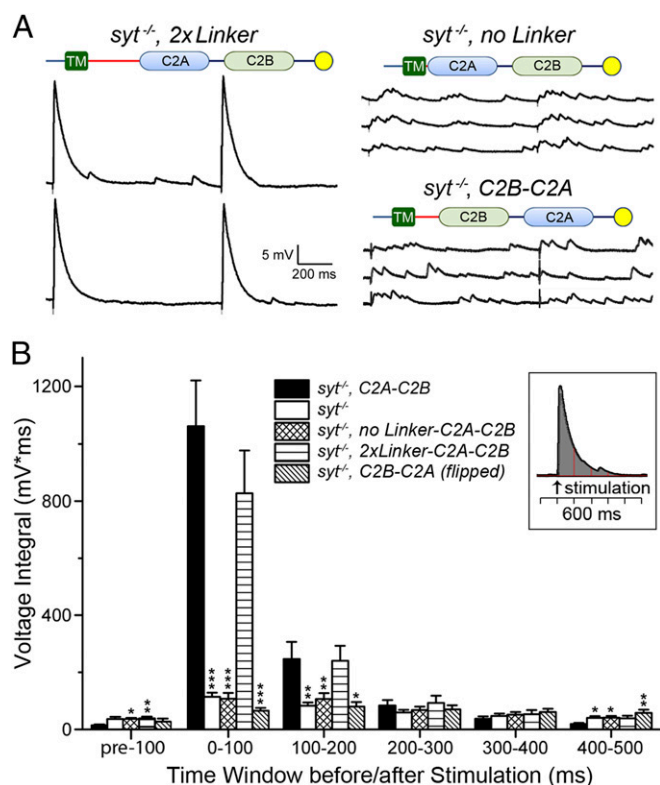
Fig. 4. Proper synaptotagmin 1 linker length and C2 domain orientation are required for synchronous release. (*A*) Representative EJPs for *syt1*$^{-/-}$ mutants rescued with the indicated transgenic constructs: (*Left*) 2× linker; (*Right*) no linker and C2B-C2A. (Scale bar: 5 mV and 200 ms.) (*B*) Voltage integral (mV × ms) values plotted for the indicated time bins pre- and poststimulation. Mean ± SEM are indicated. The numbers of larva examined were as follows: *syt*$^{-/-}$, C2A-C2B (WT), 10; *syt*$^{-/-}$, 12; *syt*$^{-/-}$, no Linker-C2A-C2B, 11; *syt*$^{-/-}$, 2× Linker-C2A-C2B, 7; *syt*$^{-/-}$, C2B-C2A (flipped), 5. *P < 0.05; **P < 0.01; ***P < 0.001, one-way ANOVA for WT vs. genotypes indicated at a 100-ms interval.

Syt1 induced a similar enhancement of the slower asynchronous phase of release (Fig. 2). Precisely how the aspartate-to-asparagine C2 domain mutants used in our study affect lipid interactions in vivo is unclear, considering that they could potentially trigger enhanced Ca^{2+}-independent lipid interactions that would activate asynchronous release. However, similar mutations in the normal synaptic vesicle-tethered version failed to induce enhanced asynchronous release, indicating that this property is unique to the cytoplasmic (and plasma membrane-tethered) versions of Syt1.

An alternative model to account for the enhanced asynchronous release under these conditions is that cytoplasmic Syt1 supports docking and endocytosis that is defective in the null mutant, leading to an increased number of vesicles that can be activated by the asynchronous Ca^{2+} sensor. Although we cannot completely exclude this possibility, our previous result with Ca^{2+}-binding-defective full-length Syt1 (C2A*-C2B*) is inconsistent with this model (21). The full-length C2A*-C2B* Syt1 could not support synchronous release, but restored normal synaptic vesicle number and vesicle docking, as quantified by EM (21); however, this mutated version of full-length Syt1 does not show the enhanced asynchronous release induced by the cytoplasmic and plasma membrane-tethered versions (Fig. 2), suggesting that mechanisms outside of vesicle docking and endocytosis may be relevant.

We also found a requirement for a specific linker domain length to connect the C2 domains to a synaptic vesicle. Although *syt1*$^{-/-}$ mutants rescued with a 2× linker were indistinguishable from those rescued with the WT counterpart, a shorter linker

domain did not support synchronous fusion (Fig. 4). How flexible the 2× linker is in vivo is unknown, but the data indicate that Syt1 might not be required to "pull" the synaptic vesicle toward the plasma membrane as a mechanism to bring the two bilayers in close proximity. Our data also indicate the specific C2 domain order in Syt1 (C2A-C2B) is important for synaptic transmission (Fig. 4), suggesting that cooperative interactions by the two C2 domains may have a spatial requirement for driving fusion. Given that plasma membrane-tethered Syt1 also fails to support synchronous evoked release and induces enhanced asynchronous release (Fig. 3), our data indicate that Syt1 must be tethered specifically to synaptic vesicles to support Ca^{2+}-dependent, fast synchronous release in vivo at *Drosophila* synapses.

Our results differ somewhat from observations using lentivirus rescue of mouse *syt1* knockout neurons with a growth associated protein 43 (GAP43) palmitoylation domain version of Syt1 that tethers the protein to the plasma membrane (although a small amount of vesicular targeting remains with this construct; ref. 33). This plasma membrane version of Syt1 rescues peak evoked amplitude at excitatory synapses (33), but fails to fully rescue the total charge transfer at inhibitory synapses (37), indicating there may be milder kinetic differences at mammalian synapses as well. Whether myr-Syt1 and GAP43-Syt1 have differences in the efficacy of synaptic vesicle vs. plasma membrane targeting, or whether these differences reflect species-specific Syt1 requirements, will require a further study.

Besides the effects on asynchronous release, synaptic vesicle-tethered Syt1 was also required for regulation of spontaneous fusion. The cytoplasmic domain of Syt1 has been shown to form a complex with SNARE proteins in a Ca^{2+}-independent manner in vitro (43–45) and to arrest partially assembled trans-SNARE complexes before fusion (38), which may explain a potential role of Syt1 as a clamp for spontaneous release. However, our results indicate that the ability of cytoplasmic Syt1 to clamp fusion in in vitro assays (38) does not translate into an in vivo clamping effect. We observed a significant increase in spontaneous fusion events in the presence of the cytoplasmic Syt1 compared with WT-rescued or null synapses (Fig. S2), suggesting that vesicular tethering of Syt1 is also critical for its clamping function.

One of the most striking findings of our analysis is that membrane anchoring of Syt1 to synaptic vesicles defines the responsiveness and kinetics for its C2 domains to trigger vesicle fusion. How does the role of Syt1 compare with other putative Ca^{2+} sensors? Synaptotagmin 7 (Syt7), another member of Syt family that has been localized to the plasma membrane, was recently implicated in asynchronous release. Knockdown of Syt7 selectively reduced asynchronous neurotransmitter release at zebrafish neuromuscular synapses and in cultured hippocampal neurons, suggesting that Syt7 may act as a plasma membrane Ca^{2+} sensor for asynchronous fusion (46, 47). Whether Syt7 and myr-Syt1 share common effector interactions to trigger asynchronous release is unclear; however, unlike the observation with Ca^{2+}-binding-defective cytoplasmic Syt1, Syt7 does require Ca^{2+} binding to function as an asynchronous sensor (47). In addition, a potential similarity of cytoplasmic Syt1 to Doc2, a cytoplasmic Ca^{2+} sensor protein family recently implicated in asynchronous and spontaneous vesicle fusion, can be seen (48). The Doc2s are a family of cytoplasmic proteins (α, β, γ) that contains dual C2 domains capable of binding to phospholipids in a Ca^{2+}-dependent manner (49). Although *Drosophila* lacks a Doc2 homolog, mammalian studies of the protein family suggest that it regulates synaptic vesicle release without membrane tethering (48). As such, subcellular localizations of Ca^{2+} sensors, together with their Ca^{2+}-binding properties and effector interactions, are likely key determinants of the speed of synaptic vesicle exocytosis.

In summary, we conclude that tethering of Syt1 to synaptic vesicles in vivo is a prerequisite for its role in facilitating fast

synchronous synaptic vesicle release and suppressing asynchronous and spontaneous fusion.

Materials and Methods

***Drosophila* Stocks and Genetics.** *Drosophila melanogaster* male larvae and adult flies were cultured on standard medium at 22 °C. Generation of transgenic Syt1 lines is detailed in *SI Materials and Methods*.

Western Blot and Immunohistochemistry Analyses. Western blot analyses were performed with 1~3-d-old adult fly heads as described previously (21). The primary antibodies used included rabbit anti-synaptogyrin (gyr;1:20,000) and mouse anti-myc (1:500; Life Technologies) or anti-His antibodies (1:1,000; Qiagen). Immunohistochemistry on third instar larvae was performed with

Syt1 (1:500) and Syx antisera (1:100), followed by FITC- and rhodamine red–conjugated secondary antibodies (1:250; Life Technologies). The procedures are described in more detail in *SI Materials and Methods*.

Electrophysiology. Intracellular recordings of EJPs and mEJPs were performed as described previously (21) at muscle fiber 6 of segments A3–A5, using HL3.1 saline at fixed $[Ca^{2+}]$ (1.0 for EJPs and 0.2 mM for mEJPs). Data acquisition and analysis are described in *SI Materials and Methods*.

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