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

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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\textsuperscript{2+}-dependent manner and can substitute for full-length Syt1 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 \textit{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.

Significance

Synaptotagmin 1 (Syt1) is widely considered to act as the fast Ca\textsuperscript{2+} sensor for synchronous synaptic vesicle fusion through its tandem Ca\textsuperscript{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.

Author contributions: J.L. and J.T.L. designed research; J.L. performed research; J.L. analyzed data; and J.L. and J.T.L. wrote the paper.

The authors declare no conflict of interest.

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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 2x 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 FCo31 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\(^{AD1}\)/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...
null mutants rescued with WT, 2
null mutants comparable to their WT coun-
trpart (Fig. 4A). The no linker version
mutants rescued with virtually no linker failed to restore normal
synchronous release observed with cytoplasmic Syt1. These results
indicate that tethering of Syt1 to synaptic vesicles is indispens-
able 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). How-
ever, 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 tether-
ing to synaptic vesicles in vivo. We generated a transgenic con-
struct in which the N-terminal region of Syt1, including the trans-
membrane domain, was replaced with a myristoylation motif
(myr-C2A-C2B or myr-Syt1 hereinafter), a lipid anchor that has
been successfully used in Drosohila to target other proteins to the
synaptic plasma membrane in vivo (41) (Fig. 3A). Endogenous Syt1
displays a characteristic halo-like distribution pattern at syn-
apses that corresponds to synaptic vesicles distributed throughout
the bouton, including the interior of the terminal (Fig. 3B, Upper,
arrowheads). In contrast, myr-Syt1 was localized at the periphery
of synaptic terminals (Fig. 3B, Lower, arrows), with increased co-
localization with syntaxin (Syx), a plasma membrane t-SNARE
protein [Fig. 3C; 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. 0.56 ± 0.02 for myr-Syt1, P < 0.05].

We next assayed the ability of myr-Syt1 to restore synaptic
responses in syt\(^{-/-}\) mutants. In contrast to WT, myr-Syt1 failed to
restore synchronous release and clamp spontaneous fusion (Fig. 3C,
Fig. 3S, Table S2). In addition, myr-Syt1 resulted in a significant increase in asynchronous vesicle release, a pattern indistinguis-
able from that observed with cytoplasmic Syt1 (Fig. 3D, 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 neuro-
transmitter 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 (2x linker) rescued synaptic trans-
mission defects in syt1\(^{-/-}\) mutants comparable to their WT coun-
trpart (Fig. 4A and B). In contrast to the extend linker, syt1
mutants rescued with virtually no linker failed to restore normal
spontaneous evoked responses, with responses indistinguishable from those of
syt1\(^{-/-}\) (Fig. 4A and B). We did not detect any significant differ-
ces in asynchronous responses occurring between 200 and 500 ms
poststimulation among syt1\(^{-/-}\) mutants rescued with WT, 2x 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. 4B and Table S3). These results indicate that
the linker domain has a minimal length requirement to facilitate
synchronous release and to clamp spontaneous fusion.

not enhance asynchronous release (Fig. 2B). 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 cyto-
plasmic 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 presti-
mulation voltage integral in cytoplasmic Syt1-expressing animals fell far short of explaining the robust increase in stimulation-
induced asynchronous release (Fig. 2B). 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. 2B). Thus, en-
hanced spontaneous release cannot explain the increased asyn-
chronous fusion observed with cytoplasmic Syt1. These results
indicate that tethering of Syt1 to synaptic vesicles is indispens-
able for its function to selectively facilitate fast synchronous
vesicle fusion.

Enhancement of asynchronous release by cytoplasmic synap-
tagmin 1. (A) Representative traces of consecutive EJPs recorded in HL3.1
saline with 1.0 mM extracellular [Ca\(^{2+}\)] shown for syt1 null mutants (syt1\(^{-/-}\)
rescued with the indicated transgenic constructs. (Scale bars: 5 mV and
200 ms). (B) Voltage integral (mV x ms) values from EJP responses plotted
for the indicated time bins pre- and poststimulation for the specified gen-
yotypes. (Inset) Calculation of voltage integral in 100-ms bins (red vertical
lines). Mean ± SEM are indicated. The numbers of larvae examined were as
follows: syt1\(^{-/-}\), C2A-C2B (WT), 10; syt1\(^{-/-}\), 12; syt1\(^{-/-}\), cytoplasmic C2A-C2B, 7; syt1\(^{-/-}\),
cytoplasmic C2A*-C2B*, 4; syt1\(^{-/-}\), 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.
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<sup>−/−</sup> 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<sup>−/−</sup> mutants (Fig. 4A 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. 4B 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<sup>2+</sup>-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<sup>2+</sup>-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 they 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<sup>−/−</sup> 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<sup>−/−</sup> (Fig. 2B). 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<sup>2+</sup>-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<sup>2+</sup>-binding-defective cytoplasmic
Syt1 induced a similar enhancement of the slower asynchronous phase of release (Fig. 2). Precisely how the aspartate-to-asparagine C2 domain mutations 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 syt\(^{1−/−}\) mutants rescued with a 2x linker were indistinguishable from those rescued with the WT counterpart, a shorter linker domain did not support synchronous fusion (Fig. 4). How flexible the 2x 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 vitro (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 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 1–3–5–old adult fly heads as described previously (21). The primary antibodies used included rabbit anti-synaptogyrin (1:1,200, 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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