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Genetic Analysis of Synaptotagmin C2 Domain Specificity in Regulating Spontaneous and Evoked Neurotransmitter Release

Jihye Lee, Zhuo Guan, Yulia Akbergenova, and J. Troy Littleton

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Synaptic vesicle fusion mediates communication between neurons and is triggered by rapid influx of Ca$^{2+}$. The Ca$^{2+}$-triggering step for fusion is regulated by the synaptic vesicle transmembrane protein Synaptotagmin 1 (Syt1). Syt1 contains two cytoplasmic C2 domains, termed C2A and C2B, which coordinate Ca$^{2+}$ binding. Although C2A and C2B share similar topology, binding of Ca$^{2+}$ ions to the C2B domain has been suggested as the only critical trigger for evoked vesicle release. If and how C2A domain function is coordinated with C2B remain unclear. In this study, we generated a panel of Syt1 chimeric constructs in order to delineate the unique and shared functions of each C2 domain in regulation of synaptic vesicle fusion. Expression of Syt1 transgenes containing only individual C2 domains, or dual C2A-C2A or C2B-C2B chimeras, failed to restore Syt1 function in a syt1$^{-/-}$ null mutant background, indicating both C2A and C2B are specifically required to support fast synchronous release. Mutations that disrupted Ca$^{2+}$ binding to both C2 domains failed to rescue evoked release, but supported synaptic vesicle docking and endocytosis, indicating that these functions of Syt1 are Ca$^{2+}$-independent. The dual C2 domain Ca$^{2+}$-binding mutant also enhanced spontaneous fusion while dramatically increasing evoked release when co-expressed with native Syt1. Together, these data indicate that synaptic transmission can be regulated by Syt1 multimerization and that both C2 domains of Syt1 are uniquely required for modulating Ca$^{2+}$-independent spontaneous fusion and Ca$^{2+}$-dependent synchronous release.

Introduction

Neurotransmitter release at synapses occurs within milliseconds following Ca$^{2+}$ influx through voltage-gated channels (Llinás et al., 1981; Neher and Zucker, 1993; Sabatini and Regehr, 1996; Sun and Wu, 2001; Wojcik and Brose, 2007). Although the full complement of Ca$^{2+}$ binding proteins that coordinate synaptic vesicle fusion is unknown, the synaptic vesicle protein Synaptotagmin 1 (Syt1) has emerged as a key Ca$^{2+}$ sensor that regulates fast synchronous release. Syt1 consists of a short intraluminal N-terminal region, a single transmembrane domain, and two cytoplasmic PKC-homologous repeats (C2 domains) that bind Ca$^{2+}$ via negatively charged aspartate residues (Perin et al., 1990; Perin et al., 1991; Sutton et al., 1995; Ubach et al., 1998; Desai et al., 2000). In vitro studies have demonstrated Ca$^{2+}$-dependent interactions between Syt1 and plasma membrane phospholipids (Brose et al., 1992; Chapman and Jahn, 1994; Sutton et al., 1995; Fernandez et al., 2001), as well as the neuronal SNARE complex (Chapman et al., 1995; Zhang et al., 2002). The loss of Syt1 severely impairs Ca$^{2+}$-dependent synchronous vesicle release (Geppert et al., 1994; Geppert et al., 1997; Voets et al., 2001; Yoshihara and Littleton, 2002; Nishiki and Augustine, 2004b; Liu et al., 2009).

To determine how Syt1 regulates fusion, several studies have focused on the function of its two C2 domains, termed C2A and C2B. Despite the robust interaction between the C2A domain and membrane phospholipids (Chapman and Davis, 1998; Bai et al., 2000; Bai et al., 2002; Stevens and Sullivan, 2003; Paddock et al., 2008), neutralization of negatively charged aspartate residues (D to N) in C2A does not disrupt synchronous neurotransmitter release (Fernández-Chacón et al., 2002; Robinson et al., 2002; Stevens and Sullivan, 2003). In contrast, a D229E substitution in the Drosophila C2A domain reduced vesicle fusion (Striegel et al., 2012), whereas neutralization of D232 (D232N) in mice enhanced synaptic transmission (Fernández-Chacón et al., 2002; Stevens and Sullivan, 2003; Pang et al., 2006a). Together, these results suggest that the C2A domain may regulate exocytosis, but its specific function remains unclear. In contrast to C2A, Ca$^{2+}$ binding to the C2B domain is required for synchronous release (Littleton et al., 1994; Littleton et al., 2001; Mackler et al., 2002; Nishiki and Augustine, 2004a; Nishiki and Augustine, 2004b; Shin et al., 2009; Yoshihara et al., 2010).

Although these studies emphasize the importance of Ca$^{2+}$-binding to C2B, there is no experimental evidence to indicate that...

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C2B can function without C2A. Indeed, membrane penetration by Syt1 requires cooperativity between its tandem C2 domains (Bai et al., 2002; Herrick et al., 2006). Here, we tested the function of each C2 domain and identified cooperative actions of C2A and C2B that regulate release at Drosophila neuromuscular junctions (NMJs). We generated transgenic Syt1 constructs bearing Ca\(^{2+}\) -binding mutations in each C2 domain or that expressed proteins with chimeric C2 domain structure. Our results indicate that the essential function for the C2B domain in fusion requires C2A.

In addition, similar interactions are required for regulation of spontaneous vesicle release, suggesting that Syt1 C2 domain cooperativity regulates multiple synaptic vesicle release pathways.

Materials and Methods

Drosophila stocks and genetics. Drosophila melanogaster were cultured on standard medium at 22°C. Female larvae and adult flies were used for analyses described below unless indicated. DNA constructs for UAS-synaptotagmin 1 (syt1) C2A\(^{-}\)D3/4N encoding Syt1\(^{D16N, D18N}\) (C2A\(^{-}\)C2B\(^{+}\)) were obtained from N.E. Reist (Colorado State University, Fort Collins, CO). DNA for UAS-syt1 C2A\(^{-}\)D3/4N encoding Syt1\(^{D282N, D284N}\) (C2A\(^{-}\)C2B\(^{-}\)) was generated using the QuikChange multiSite-Directed Mutagenesis Kit (Stratagene) with the primer cctgtgtttgccattttcAacttcAatcgc. A C2B\(^{-}\) was generated using the QuikChange multiSite-Directed Mutagenesis Kit (Stratagene) with the primer ctcgtgtttgccattttcAacttcAatcgc. A similar strategy was used to generate UAS-syt1 C2A\(^{-}\)D3/4N-C2B-D3/4N (C2A\(^{-}\)C2B\(^{+}\)) encoding Syt1\(^{D282N, D284N}\) (C2A\(^{-}\)C2B\(^{-}\)) was generated using the QuikChange multiSite-Directed Mutagenesis Kit (Stratagene) with the primer ctcgtgtttgccattttcAacttcAatcgc. A similar strategy was used to generate UAS-syt1 C2A\(^{-}\)D3/4N-C2B-D3/4N (C2A\(^{-}\)C2B\(^{+}\)) encoding Syt1\(^{D282N, D284N}\) (C2A\(^{-}\)C2B\(^{-}\)) was generated using the QuikChange multiSite-Directed Mutagenesis Kit (Stratagene) with the primer ctcgtgtttgccattttcAacttcAatcgc. A similar strategy was used to generate UAS-syt1 C2A\(^{-}\)D3/4N-C2B-D3/4N (C2A\(^{-}\)C2B\(^{+}\)) encoding Syt1\(^{D282N, D284N}\) (C2A\(^{-}\)C2B\(^{-}\)) was generated using the QuikChange multiSite-Directed Mutagenesis Kit (Stratagene) with the primer ctcgtgtttgccattttcAacttcAatcgc. A similar strategy was used to generate UAS-syt1 C2A\(^{-}\)D3/4N-C2B-D3/4N (C2A\(^{-}\)C2B\(^{+}\)) encoding Syt1\(^{D282N, D284N}\) (C2A\(^{-}\)C2B\(^{-}\)) was generated using the QuikChange multiSite-Directed Mutagenesis Kit (Stratagene) with the primer ctcgtgtttgccattttcAacttcAatcgc. A similar strategy was used to generate UAS-syt1 C2A\(^{-}\)D3/4N-C2B-D3/4N (C2A\(^{-}\)C2B\(^{+}\)) encoding Syt1\(^{D282N, D284N}\) (C2A\(^{-}\)C2B\(^{-}\)) was generated using the QuikChange multiSite-Directed Mutagenesis Kit (Stratagene) with the primer ctcgtgtttgccattttcAacttcAatcgc. A similar strategy was used to generate UAS-syt1 C2A\(^{-}\)D3/4N-C2B-D3/4N (C2A\(^{-}\)C2B\(^{+}\)) encoding Syt1\(^{D282N, D284N}\) (C2A\(^{-}\)C2B\(^{-}\)).

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Results

Generation of Syt1 transgenic constructs defective in Ca\(^{2+}\) binding.

It has been well established that five conserved aspartate residues (termed D1-D5) form a Ca\(^{2+}\) -binding pocket within each C2 domain (Fig. 1A, asterisks; Shao et al., 1996; Sutton et al., 1999; Fernandez et al., 2001). Neutralization of these residues leads to a...
loss of Ca$^{2+}$ binding and subsequent disruption in Syt1 function (Littleton et al., 2001; Fernández-Chacón et al., 2002; Mackler et al., 2002; Stevens and Sullivan, 2003). Although the C2B domain is critical for activating synchronous vesicle release (Littleton et al., 1994; Littleton et al., 2001; Mackler et al., 2002; Nishiki and Augustine, 2004a; Nishiki and Augustine, 2004b Shin et al., 2009; Augustine, 2004a; Nishiki and Augustine, 2004b Shin et al., 2009; Littleton et al., 2001; Fernández-Chacón et al., 2002; Mackler et al., 2002; Stevens and Sullivan, 2003), there are no experimental data to indicate that the C2B domain can function as the sole Ca$^{2+}$-binding module independent of C2A. To delineate isolated functions conveyed by each C2 domain versus cooperative interactions requiring both C2 domains, we generated transgenic Syt1 constructs bearing mutations in the key aspartate residues in each C2 domain in Drosophila (Fig. 1B). The five conserved aspartate residues involved in binding Ca$^{2+}$ are highly conserved across the phyla (Fig. 1A, asterisks). Similar to prior approaches to eliminate Ca$^{2+}$ binding (Littleton et al., 2001; Fernández-Chacón et al., 2002; Mackler et al., 2002; Stevens and Sullivan, 2003), two of the five residues in each C2 domain were mutated to asparagines (Fig. 1A, red boxes; D282/284N and in C2A and D416/418N in C2B). This allowed generation of mutants disrupting Ca$^{2+}$ binding to C2A, C2B, or both C2 domains. These mutations (indicated hereafter as C2A*, C2B*, or C2A*-C2B*) were introduced into transgenic constructs (Fig. 1B, left) and expressed in the syt1 null mutant (syt1$^{-/-}$) background using the GAL4-UAS system (Brand and Perrimon, 1993).

To begin analyzing these transgenic animals, we first examined expression of the mutant protein in the syt1$^{-/-}$ background to determine whether they localized properly at NMJs, similar to endogenous Syt1. Immunoreactivity against Syt1 indicated that the mutant proteins were distributed at NMJs in a pattern similar to that of endogenous Syt1 (Fig. 2A), suggesting that Ca$^{2+}$ binding to the C2 domains is not required for synaptic targeting of Syt1. To avoid complications in functional analysis that might originate from differential expression of each construct, transgenic lines with similar expression levels by Western analysis (Fig. 2B) were chosen for comparison in subsequent experiments.

**Figure 1.** Syt1 transgenic constructs and sequence similarity. A. Amino acid sequences of human, mouse, and Drosophila Syt1 are compared. A single transmembrane domain and two C2 domains, C2A and C2B, are indicated as dark green, blue, and light green blocks, respectively. The five aspartate residues (D) involved in binding Ca$^{2+}$ ions in each C2 domain are indicated with red asterisks. The third and fourth of these five aspartate residues were mutated to asparagines (N) to disrupt Ca$^{2+}$ binding (Littleton et al., 2001; Fernández-Chacón et al., 2002; Mackler et al., 2002; Stevens and Sullivan, 2003), two of the five conserved aspartate residues in the C2B domain are highly conserved across the phyla (Fig. 1A, asterisks). Similar to prior approaches to eliminate Ca$^{2+}$ binding (Littleton et al., 2001; Fernández-Chacón et al., 2002; Mackler et al., 2002; Stevens and Sullivan, 2003), two of the five aspartate residues in the C2B domain were mutated to asparagines (Fig. 1A, red boxes; D282/284N and in C2A and D416/418N in C2B). This allowed generation of mutants disrupting Ca$^{2+}$ binding to C2A, C2B, or both C2 domains. These mutations (indicated hereafter as C2A*, C2B*, or C2A*-C2B*) were introduced into transgenic constructs (Fig. 1B, left) and expressed in the syt1 null mutant (syt1$^{-/-}$) background using the GAL4-UAS system (Brand and Perrimon, 1993).

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**Essential role of Ca$^{2+}$ binding to the Syt1 C2B domain for synchronous neurotransmitter release**

To functionally characterize the role of Ca$^{2+}$ binding to each C2 domain in neurotransmitter release, we measured nerve-evoked excitatory junction potentials (eEJPs) at third instar NMJs in the presence of low (0.2 mM) and high (1.0 mM) external [Ca$^{2+}$] (Fig. 3A, upper and lower traces, respectively). We found that synchronous release at lower Ca$^{2+}$ levels (0.2 mM) was restored more effectively in null mutants rescued with Syt1 lacking C2A Ca$^{2+}$ binding (C2A*-C2B) than those with full-length wild-type Syt1 (C2A-C2B) (Fig. 3B, left, p < 0.01), similar to observations made at Drosophila embryonic NMJs (Yoshihara et al., 2010). Despite differences in the degree of rescue at a lower Ca$^{2+}$ level, both C2A-C2B and C2A*-C2B Syt1 constructs restored synchronous release to near wild-type levels at more physiological Ca$^{2+}$ levels (Fig. 3A, bottom traces in each pair; Fig. 3B, right). However, as previously observed (Littleton et al., 2001; Fernández-Chacón et al., 2002; Mackler et al., 2002; Stevens and Sullivan, 2003), Syt1 with defective C2B Ca$^{2+}$ binding (C2A*-C2B*) failed to rescue synchronous release, regardless of the presence of a wild-type or mutated C2A domain (C2A-C2B* and C2A*-C2B*) or external Ca$^{2+}$ concentration (0.2 and 1.0 mM) (Fig. 3A, B; p < 0.01 at 0.2 mM [Ca$^{2+}$] and p < 0.001 at 1.0 mM). As a result, the ratio of eEJP levels (0.2 mM) was restored more effectively in null mutants rescued with Syt1 lacking C2A Ca$^{2+}$ binding (C2A*-C2B) than those with full-length wild-type Syt1 (C2A-C2B) (Fig. 3B, left, p < 0.01), similar to observations made at Drosophila embryonic NMJs (Yoshihara et al., 2010). Despite differences in the degree of rescue at a lower Ca$^{2+}$ level, both C2A-C2B and C2A*-C2B Syt1 constructs restored synchronous release to near wild-type levels at more physiological Ca$^{2+}$ levels (Fig. 3A, bottom traces in each pair; Fig. 3B, right). However, as previously observed (Littleton et al., 2001; Fernández-Chacón et al., 2002; Mackler et al., 2002; Stevens and Sullivan, 2003), Syt1 with defective C2B Ca$^{2+}$ binding (C2A*-C2B*) failed to rescue synchronous release, regardless of the presence of a wild-type or mutated C2A domain (C2A-C2B* and C2A*-C2B*) or external Ca$^{2+}$ concentration (0.2 and 1.0 mM) (Fig. 3A, B; p < 0.01 at 0.2 mM [Ca$^{2+}$] and p < 0.001 at 1.0 mM). As a result, the ratio of synaptic failure, defined as no detectable eEJP following nerve stimulation, was comparable between syt1$^{-/-}$ and C2A-C2B or wild-type Syt1 rescued larvae at a higher Ca$^{2+}$ level (1.0 mM) (Fig. 3C). Together, these data support the model that Ca$^{2+}$ binding to C2B, but not C2A, is essential for fast synchronous neurotransmitter release.
Cooperativity between Syt1 C2 domains in synchronous neurotransmitter release

Rescue data indicate that the C2B domain is the major Ca\(^{2+}\)-binding module involved in regulation of synchronous fusion. However, these results do not exclude a contribution from the C2A domain in C2B-driven Ca\(^{2+}\)-dependent exocytosis. Given that mutations in the C2B domain (C2A-C2B\(^*\)) led to a complete failure to rescue, regardless of the presence of a wild-type or Ca\(^{2+}\)-binding defective C2A domain (Fig. 3, C2A-C2B\(^*\) and C2A*-C2B\(^*\)), it is possible that C2B functions as a major Ca\(^{2+}\)-binding module without a C2A domain present. To assay the function of each C2 domain separately, we created transgenic constructs that deleted the entire C2A or C2B domain. In addition, we created chimeric transgenic lines that expressed a dual C2A-C2A or C2B-C2B Syt1 protein where the corresponding endogenous C2 domain was replaced to generate a homologous C2 domain architecture (Fig. 1B, right). A construct lacking the entire C2B domain (C2A only) was properly localized at NMJs, albeit at a lower level, when detected with anti-Syt1 antibodies. However, the counterpart lacking the C2A domain (C2B only) was not recognized by immunocytochemistry (data not shown), possibly because of a lack of antigenicity to C2B epitopes with our anti-Syt1 antisera. To circumvent this problem, the dual C2A-C2A and C2B-C2B constructs were tagged with hemagglutinin. These constructs were detected at NMJs at similar patterns to endogenous Syt1 when visualized with an anti-hemagglutinin antibody (data not shown).

We first examined whether a functional C2A domain in the absence of C2B could restore release in syt1\(^{-/-}\). Syt1 transgenes expressing C2A in the absence of C2B failed to rescue fast synchronous release, even in high extracellular [Ca\(^{2+}\)] (1.0 mm, Fig. 4A, B, p < 0.001). A similar failure to rescue was also observed in the case of Syt1 transgenes expressing C2A that lacks Ca\(^{2+}\)-binding ability (C2A\(^*\), data not shown). To determine whether the inability of C2A to restore release was due to the lack of a C2B domain or simply due to the requirement for the dual C2 domain structure found in all syntaptotagmin isoforms (Sudhof and Rizo, 1996), we examined transgenic strains that replaced the C2B domain with a second C2A to retain the characteristic twin C2 domain structure (Figs. 1B, right, 4A, C2A-C2A). We observed that C2A-C2A transgenic rescue lines had no ability to restore release, as the amplitude of eEJPs remained similar to that in syt1\(^{-/-}\) and far below control levels (Fig. 4B, p < 0.001). These data indicate that the C2A domain alone, as a dual C2A-C2A module, cannot support fast synchronous release.

We next assayed whether C2B alone was sufficient to support synchronous release when expressed in syt1\(^{-/-}\) mutants. Constructs expressing a Syt1 construct lacking the C2A domain were not capable of restoring synchronous release, regardless of the Ca\(^{2+}\)-binding ability of C2B (Fig. 4A,B, p < 0.001; data not shown for C2B\(^*\)). We next examined whether a transgenic protein expressing dual C2B domains (C2B-C2B) was able to function in vesicle fusion. Synchronous release in syt1\(^{-/-}\) larvae expressing this construct was similar to that observed in syt1\(^{-/-}\)-alone (Fig. 4A), indicating that C2B cannot function in the absence of C2A (Fig. 4B, p < 0.001). In summary, these data demonstrate that Ca\(^{2+}\)-dependent exocytosis mediated by the C2B domain requires the presence of C2A, indicating that the activity of both C2 domains is specifically required to regulate synchronous synaptic vesicle fusion.

C2A and C2B function cooperatively to regulate spontaneous neurotransmitter release

In addition to evoked neurotransmitter release, Syt1 has been shown to regulate spontaneous fusion, as evidenced by the elevated rates of spontaneous release in syt1\(^{-/-}\) null mutants (Di-Antonio and Schwarz, 1994; Geppert et al., 1994; Littleton et al., 1994; Mackler et al., 2002; Yoshihara and Littleton, 2002; Pang et al., 2006b; Xu et al., 2009). However, the role of the C2A and C2B domains in regulation of spontaneous fusion has just begun to be explored (Xu et al., 2009) and remains poorly characterized. We
examined whether C2A and C2B regulate spontaneous release by measuring mEJP frequency and amplitude in syt1/H11002/H11002 larvae rescued with control and mutated transgenic Syt1 constructs (Fig. 5). Consistent with previous reports, syt1/H11002/H11002 mutants displayed a slight but significant increase in the frequency of spontaneous release (Fig. 5A, B, bottom, \(p < 0.05\)). Expression of the wild-type Syt1 transgene (C2A-C2B) in syt1/H11002/H11002 larvae restored spontaneous release to control levels (Fig. 5A, B, bottom). Syt1 transgenes containing neutralization of two aspartate residues (D3/4N) in C2A (C2A*-C2B) appeared to be more effective in clamping spontaneous release compared with those rescued with wild-type Syt1, although the effect did not reach statistical significance (Fig. 5B, bottom, \(p = 0.32\)). In contrast to C2A mutations, mEJP frequency appeared elevated in larvae expressing Ca\(^{2+}\)-binding mutations in C2B (C2A-C2B*) (Fig. 5B, bottom, \(p = 0.14\)), comparable to syt1\(^{-/-}\) (Fig. 5A, B, bottom), suggesting that spontaneous release may be differentially regulated by Ca\(^{2+}\)-binding to the C2A and C2B domains.

To determine whether the ability of C2B to regulate spontaneous fusion requires wild-type C2A, we assayed Syt1 transgenes expressing Ca\(^{2+}\)-binding mutants in both C2 domains (C2A*-C2B*). Surprisingly, expression of the C2A*-C2B* transgenic rescue in the syt1\(^{-/-}\) null background induced a dramatic increase in mEJP frequency regardless of external [Ca\(^{2+}\)] (Fig. 5A, B, bottom, \(p < 0.001\)). The elevated mini-frequency was far beyond the levels observed in syt1\(^{-/-}\) mutants alone or rescue strains expressing C2A-C2B* (Fig. 5B, \(p < 0.001\), C2A*-C2B* vs syt1\(^{-/-}\) or C2A-C2B*).

The increase in spontaneous release induced by expression of a Ca\(^{2+}\)-binding defective form of Syt1 could be secondary to
Ca\(^{2+}\)-independent roles for the protein in synaptic vesicle docking, priming, or endocytosis that enhance the number of docked synaptic vesicles. To test this possibility, we examined the distribution of synaptic vesicles in syt\(^{-/-}\) null mutants expressing C2A*-C2B* using transmission electron microscopy and compared them with null mutants alone or rescue lines expressing the wild-type Syt1 protein (C2A-C2B). As previous reports have indicated (Reist et al., 1998; Liu et al., 2009; Young and Neher, 2009), syt\(^{-/-}\) null mutants displayed a reduction in docked synaptic vesicles (Fig. 6A,D, black column, \(p < 0.001\)), as well as a reduced vesicle density, measured as the number of vesicles per unit bouton area (Fig. 6E, \(p < 0.001\)). Expression of C2A*-C2B* resulted in an increase in docked synaptic vesicles adjacent to active zones, similar to that in strains expressing the wild-type transgene (Fig. 6A,D, black columns). Likewise, vesicle density in syt\(^{-/-}\) mutants expressing C2A*-C2B* was comparable to syt\(^{-/-}\) animals rescued with the wild-type protein and significantly greater than animals lacking Syt1 altogether (Fig. 6E, \(p < 0.001\)). We conclude that a Syt1 transgene with no Ca\(^{2+}\)-binding ability (C2A*-C2B*) can partially rescue defects in synaptic vesicle docking and total vesicle number. Given that docked vesicle number is similar in syt\(^{-/-}\) mutants expressing the wild-type or C2A*-C2B* Syt1, it is unlikely that structural changes in synaptic vesicle distribution underlie the enhanced spontaneous release induced by C2A*-C2B* (Fig. 5A,B). Rather, Ca\(^{2+}\)-binding defective Syt1 appears to increase the fusogenicity of synaptic vesicles, resulting in a dramatic elevation in mEJP frequency.

Because the effects on evoked release suggested cooperative actions of the C2 domains, we investigated whether such cooperativity between C2 domains exists for the regulation of spontaneous release. We tested the role of the C2 domains by measuring mEJP frequency and amplitude in syt\(^{-/-}\) mutants expressing single or dual C2 domains in either wild-type or mutated form (C2A, C2A*, and C2A-C2A; C2B, C2B*, and C2B-C2B). mEJP frequency in larvae expressing transgenic constructs that lack C2B (C2A, C2A*, and C2A-C2A) remained comparable with syt\(^{-/-}\) mutants (Fig. 5C,D, gray dashed line) but was still far below the level of those expressing C2A*-C2B* (Fig. 5D, black dashed line, \(p < 0.001\)). Similarly, transgenic constructs consisting solely of the C2B domain (C2B, C2B*, and C2B-C2B) failed to rescue the increased mini-frequency in null mutants (Fig. 5C,D) but remained far below the level observed in syt\(^{-/-}\) rescued with C2A*-C2B* (Fig. 5D, \(p < 0.001\)). These results indicate that cooperativity between C2A and C2B is required not only for synchronous neurotransmitter release but also for Syt1 function as a partial clamp of spontaneous release. It should be noted that there are no observed Ca\(^{2+}\)-dependent changes in mEJP frequency within a range (0.2–2.0 mM) of external [Ca\(^{2+}\)] at Dro sophila NMJs (data not shown), in contrast to the Ca\(^{2+}\)-dependent nature of spontaneous release at mammalian synapses (Xu et al., 2009; Kochubej and Schneggenburger, 2011). Thus, it is likely that Syt1 exerts its function as a partial clamp in a Ca\(^{2+}\)-independent manner. Given that C2A*-C2B* lacks Ca\(^{2+}\)-binding ability and Ca\(^{2+}\)-dependent interaction with SNAREs (Earles et al., 2001; Shin et al., 2009), the mutated protein may alter Ca\(^{2+}\)-independent interactions of Syt1 with other partners regulating vesicle release, such as SNAREs and membrane phospholipids. Unlike mEJP frequency, mEJP amplitude remained similar among the different transgenic expressions, except for syt\(^{-/-}\) larvae expressing a C2B-C2B chimeric protein, which significantly enhanced mEJP amplitude (Figs. 5C, 6B, \(p < 0.001\)). The increase in mEJP amplitude in C2B-C2B–expressing animals was associated with the appearance of significantly larger synaptic vesicles by transmission electron microscopy (Fig. 6A, arrowheads) and with an increase in diameter of synaptic vesicles near active zones, compared with syt\(^{-/-}\) rescued with the wild-type Syt1 (C2A-C2B) (Fig. 6C). Furthermore, we also found a significant reduction in the number of vesicles distant from active zones in syt\(^{-/-}\) larvae expressing a C2B-C2B protein (Fig. 6D), resulting in a reduced total vesicle density similar to syt\(^{-/-}\) (Fig. 6E, \(p < 0.001\)). The Syt1 C2B domain is known to interact with several proteins involved in synaptic vesicle endocytosis (Jorgensen et al., 1995; Haucke and De Camilli, 1999; Littleton et al., 2001; Poskanzer et al., 2003; Nicholson-Tomishima and Ryan, 2004; Yao et al., 2012a; Yao et al., 2012b). Considering that the distribution of vesicles near active zones in syt\(^{-/-}\) rescued with C2B-C2B was restored to wild-type levels (Fig. 6D, black column), our findings argue that the C2B-C2B protein is capable of promoting vesicle docking similar to wild-type Syt1. In contrast, the reduction in total vesicle density, together with the increased vesicle diameter and enlarged mEJP amplitude, indicates that the
dual C2B-C2B protein likely alters the association of Syt1 with the endocytotic machinery, resulting in defects in vesicle number and size.

Enhanced neurotransmitter release induced by coexpression of native and Ca$^{2+}$-insensitive Syt1

Although expression of transgenic constructs in the syt1$^{-/-}$ null background allows characterization of their function in the absence of endogenous Syt1, overexpression of these constructs in the wild-type background can assay any dominant-negative or gain-of-function properties of the mutant proteins. Prior work demonstrated that overexpression of the Ca$^{2+}$ binding C2B mutant (C2A-C2B*) induces a decrease in evoked neurotransmitter release at Drosophila larval NMJs (Mackler et al., 2002). We observed a similar effect caused by overexpression of C2A-C2B* using elavC155-GAL4, which resulted in a significant decrease in eEJP amplitude compared with overexpression of wild-type Syt1 (C2A-C2B) (Fig. 7A, $p < 0.001$). Such dominant-negative effects on synchronous release were accompanied by an increase in asynchronous release (Fig. 7A, arrows) and enhanced spontaneous fusion (Fig. 7B, bottom), presumably because of the altered nature of the mutated Syt1 C2B domain interaction with membrane phospholipids (Paddock et al., 2011).

Whereas overexpression of wild-type or C2A Ca$^{2+}$ binding Syt1 mutants (C2A*-C2B) did not alter EJP amplitude at 0.2 mM extracellular [Ca$^{2+}$], overexpression of the C2A*-C2B* protein that completely lacks Ca$^{2+}$-binding ability induced a dramatic increase in EJP amplitude (Fig. 7A, $p < 0.001$) without affecting asynchronous release or spontaneous fusion (Fig. 7B). Such enhancement in synchronous release could reflect an increase in the number of synaptic vesicles available for immediate release. To test this hypothesis, we examined the synaptic ultrastructure of larvae expressing wild-type or C2A*-C2B* Syt1 (Fig. 8A, B). We found no discernible abnormalities associated with overexpression of C2A*-C2B*, including no significant increase in the number of synaptic vesicles near active zones (Fig. 8B). Indeed, the
total number of synaptic vesicles residing within a 200 nm radius of active zones was mildly reduced in larvae overexpressing Ca\(^{2+}\)-binding defective C2 domains (C2A*-C2B*) or with dual C2B domains (C2B-C2B). Boxed regions in each micrograph are magnified 2.5 \(\times\) in insets. Synaptic vesicles with abnormally large diameters are indicated with arrowheads. Scale bar, 500 nm. B, Summary of the mean mEJP amplitude is shown for syt\(^{-/-}\) as well as syt\(^{-/-}\) rescued with wild-type Syt1 (C2A-C2B), Ca\(^{2+}\)-binding defective C2A*-C2B*, or dual C2A/C2B domains. Data for syt\(^{-/-}\) as well as syt\(^{-/-}\), C2A-C2B and syt\(^{-/-}\), C2A*-C2B* from Figure 5 are presented for comparison. Data are mean \(\pm\) SEM. ***p < 0.001 and *p < 0.05, one-way ANOVA with multiple comparisons using the Fisher’s LSD test between syt\(^{-/-}\) rescued with the C2A-C2B and the indicated genotypes. The number of NMJs examined for each genotype is listed in Figure 5. C, Cumulative diameter distributions of synaptic vesicles residing within a 100 nm radius of active zones are shown for syt\(^{-/-}\) as well as those rescued with wild-type, Ca\(^{2+}\)-binding defective C2 domains, or dual C2B domains. A boxed inset is provided for a detailed comparison. D, E, The number of synaptic vesicles near or in the vicinity of active zones (D) and total synaptic vesicle density (E) are summarized for each genotype indicated. Data are mean \(\pm\) SEM. ***p < 0.001 and **p < 0.01, one-way ANOVA with multiple comparisons using the Fisher’s LSD test between syt\(^{-/-}\) rescued with the C2A-C2B and the indicated genotypes. Number of active zones analyzed for D and E: syt\(^{-/-}\), 24 and 14; syt\(^{-/-}\), C2A-C2B, 17 and 14; syt\(^{-/-}\), C2A*-C2B*, 13 and 5; and syt\(^{-/-}\), C2B-C2B, 22 and 14.

We next investigated which functional aspects of the release process were modified by overexpression of C2A*-C2B*. For in-
Ca\(^{2+}\) conditions to support synchronous release. Furthermore, the paired-pulse ratio was significantly reduced in C2A*-C2B*-overexpressing larvae compared with animals overexpressing wild-type Syt1 (Fig. 7D). Given that the paired-pulse ratio reflects release probability during the first stimulation, these results indicate that overexpression of C2A*-C2B* enhances synchronous release by increasing release probability and lowering the [Ca\(^{2+}\)] required to trigger fusion.

**Multimerization of native and Ca\(^{2+}\)-binding defective Syt1**

Our results demonstrate that expression of a Ca\(^{2+}\)-binding defective Syt1 (C2A*-C2B*) protein in the absence of native Syt1 robustly increases the rate of spontaneous fusion (Fig. 5), without restoring fast synchronous release (Fig. 3). In contrast, coexpression of C2A*-C2B* and native Syt1 resulted in a large enhancement in synchronous release (Fig. 7A), with no increase in spontaneous fusion (Fig. 7B).

If Syt1 regulates synaptic release as a monomer, we predicted that a mixed population of Ca\(^{2+}\)-binding defective and native Syt1 monomers would yield one of several possibilities: (1) both synchronous and spontaneous release would remain unchanged compared with control if native wild-type monomers in a mixed population are sufficient to perform the full repertoire of Syt1 functions; (2) synchronous release will be partially disrupted with a slight elevation of spontaneous release if wild-type and Ca\(^{2+}\)-binding defective Syt1 monomers participate independently in regulating both synchronous and spontaneous release; or (3) synchronous and spontaneous release will be differentially disrupted if native and mutated Syt1 monomers contribute unequally to different modes of synaptic release, resulting in either relatively normal synchronous release with a slightly enhanced spontaneous release or impaired synchronous release with a wild-type level of spontaneous fusion. However, our results are not consistent with these predictions, given the normal rate of spontaneous release with enhanced synchronous fusion caused by coexpression of Ca\(^{2+}\)-binding defective and native Syt1 (Fig. 7A, B). These results suggest the possibility of a Syt1 hetero-multimeric complex where native Syt1 clamps spontaneous release, whereas C2A*-C2B* Syt1 promotes evoked fusion. Indeed, the synaptopotmin family has been biochemically shown to multimerize in Ca\(^{2+}\)-independent (Fukuda et al., 1999; Bai et al., 2000; Fukuda et al., 2001) and Ca\(^{2+}\)-dependent manners (Chapman et al., 1996; Damer and Creutz, 1996; Sugita et al., 1996; Osborne et al., 1999; Desai et al., 2000). Thus, we examined whether dimerization could occur between native and Ca\(^{2+}\)-insensitive Syt1,
which might underlie the enhanced synchronous release observed in larvae overexpressing C2A*-C2B*.

We purified GFP-tagged full-length Syt1 expressed in syt1/H11002/H11002 fly heads and performed pull-down assays with GST-fused wild-type (GST-C2A-C2B) or mutated (GST-C2A*-C2B*) recombinant Syt1 (Fig. 9A). Purified GFP-tagged Syt1 bound to the cytoplasmic C2 domains of Syt1 immobilized on glutathione-Sepharose beads in the absence of Ca\(^{2+}\), as visualized by GFP immunoreactivity (Fig. 9A, EGTA, 2 mM). Further multimerization of Syt1 was observed by increasing [Ca\(^{2+}\)] to 1 or 10 mM (Fig. 9A). These Ca\(^{2+}\)-independent and Ca\(^{2+}\)-dependent interactions did not differ between wild-type and C2A*-C2B* Syt1 (Fig. 9A). Similar results were obtained when we performed pull-down assays with His-tagged wild-type cytoplasmic Syt1 C2 domains to bind GST-fused wild-type or mutated recombinant Syt1. Ca\(^{2+}\)-independent binding between His-Syt1 and GST-fused Syt1 (wild-type and C2A*-C2B*) was consistently detected (Fig. 9B). As seen with endogenous Syt1 pull-down, the interaction between His-Syt1 and GST-tagged Syt1 was enhanced in response to Ca\(^{2+}\) (Fig. 9B). Together, these findings indicate that endogenous Syt1 and Ca\(^{2+}\)-binding defective Syt1 likely form hetero-multimers in the absence of Ca\(^{2+}\), which enhances the fusogenicity of synaptic vesicles in response to Ca\(^{2+}\) (Fig. 7).

**Discussion**

**Requirement of tandem Syt1 C2A-C2B domains in regulation of neurotransmitter release**

Using transgenic rescue approaches with chimeric Syt1 proteins, we demonstrate that both the C2A and C2B domains are individually required for Syt1 function in regulating vesicle release at the Drosophila NMJ. Prior studies have established that the C2B domain of Syt1 is a critical module for activating synchronous vesicle release in Drosophila (Littleton et al., 1994; Littleton et al., 2001; Mackler et al., 2002; Yoshihara et al., 2010). In contrast, the
The contribution of the C2A domain is still being elucidated (Yoshihara et al., 2010; Striegel et al., 2012). Although the activities of isolated C2 domains in their interaction with SNAREs and membrane phospholipids remain controversial in in vitro studies, growing evidence suggests synergistic actions of both C2 domains. Isolated C2 domains, either C2A or C2B, bind syntaxin and SNAP-25 less effectively than C2A-C2B constructs in response to Ca<sup>2+</sup> in in vitro binding assays (Chapman et al., 1996; Rickman and Davletov, 2003) or reconstituted vesicle fusion assays (Tucker et al., 2004). Synergistic actions of the C2 domains also occur during lipid binding, as isolated C2 domains bind poorly to liposomes made from chromaffin granule lipid extracts compared with dual C2A-C2B proteins (Damer and Creutz, 1994). Likewise, the interaction between the C2B domain and membrane phospholipids requires an intact C2A domain, even in the absence of its Ca<sup>2+</sup>- and lipid-binding abilities (Bai et al., 2002). Furthermore, Ca<sup>2+</sup>-dependent penetration of both C2 domains into reconstituted lipid bilayers appears to occur simultaneously (Hui et al., 2006). These in vitro studies indicate that cooperative actions of C2 domains regulate SNARE and lipid interactions and match our in vivo observations that evoked neurotransmitter release requires both C2 domains of Syt1.

In addition to a dual C2 domain structure, our findings indicate that C2A and C2B are uniquely required. Double C2 domain constructs containing only C2A-C2A fail to restore Syt1 function (Fig. 4), consistent with a lack of Ca<sup>2+</sup>-dependent binding to t-SNAREs in the absence of C2B (Earles et al., 2001). Similarly, Syt2 constructs lacking the C2B domain at the calyx of Held failed to support evoked fusion in the null mutant background (Kochubey and Schneggenburger, 2011). Although the C2B domain rather than C2A plays a more prominent role in evoked fusion (Fig. 3), our results also indicate that the C2B domain is not solely responsible for Syt1 function in synaptic vesicle fusion, as an isolated or dual C2B domain cannot support synaptic vesicle fusion (Fig. 4). In addition, dual C2B-C2B constructs induce additional defects in vesicle endocytosis pathways that increase vesicle size and mEJP amplitude (Fig. 6). This contrasts with the ability of purified Syt1 dual C2B domains to induce liposome aggregation and SNARE-dependent vesicle fusion comparable with the wild-type Syt1 (C2A-C2B) in vitro (Hui et al., 2011), indicating a limitation of in vitro fusion assays in recapitulating in vivo requirements for synaptic vesicle release.

### Regulation of spontaneous fusion by Syt1 C2 domains

In addition to triggering synchronous fusion, Syt1 also partially clamps spontaneous fusion in the absence of a Ca<sup>2+</sup> signal. Transgenic constructs with Ca<sup>2+</sup>-binding mutations in C2A fully rescued the elevated spontaneous fusion rate in syt1<sup>−/−</sup> null mutants, whereas those with Ca<sup>2+</sup>-binding mutations in C2B did not (Fig. 5). Prior in vitro studies have demonstrated that C2A*-C2B is indistinguishable from wild-type C2A-C2B in its binding to membrane phospholipids (Bai et al., 2002; Bai et al., 2004), whereas mutations in the C2B domain (C2A-C2B<sup>+</sup>) facilitated Ca<sup>2+</sup>-independent binding of Syt1 to phospholipids (Mackler et al., 2002). Such differences in Ca<sup>2+</sup>-dependent lipid binding may underlie the differential regulation of spontaneous release in syt1<sup>−/−</sup> mutants rescued with C2A*-C2B versus C2A-C2B. Alternatively, neutralizations of these Ca<sup>2+</sup>-binding residues may induce differential changes in C2 domain structure that influence Ca<sup>2+</sup>-independent interactions between Syt1 and SNAREs. Syt1 has been shown to form a Ca<sup>2+</sup>-independent complex with SNARE proteins in vitro (Bennett et al., 1992; Rickman and Davletov, 2003; Shin et al., 2003) and to stabilize partially assembled trans-SNARE complexes to function as a fusion clamp (Chicka et al., 2008).

Unexpectedly, we found a dramatic enhancement in the rate of spontaneous vesicle release far beyond that observed in syt1<sup>−/−</sup> null mutants when we rescued with transgenic proteins carrying mutations of Ca<sup>2+</sup>-binding residues in both C2 domains (C2A*-C2B<sup>+</sup>, Fig. 5). Unlike the Ca<sup>2+</sup>-dependent nature of spontaneous release at mammalian synapses (Xu et al., 2009; Kochubey and Schneggenburger, 2011), Ca<sup>2+</sup>-dependent changes in mEJP frequency within a compatible
Physiological significance of Syt1 multimerization

Although the Syt family can form multimers in vitro, the relevance of such multimeric complexes on synaptic transmission in vivo has remained unclear. Different Syt family members can form homodimers via their N-terminal cysteine residues in a Ca\(^{2+}\)-independent manner (Fukuda et al., 1999; Bai et al., 2000; Fukuda et al., 2001), whereas Ca\(^{2+}\)-dependent self-association and formation of heterodimers between different Syt family members are linked to activity of the C2B domain (Chapman et al., 1996; Damer and Creutz, 1996; Sugita et al., 1996; Osborne et al., 1999; Desai et al., 2000). Genetic analyses in Drosophila have suggested Syt oligomerization may regulate synaptic transmission. Mutations in the C2B domain that alter Syt1 oligomerization (Syt\(^{AD3}\)) (DiAntonio and Schwarz, 1994; Littleton et al., 1994; Fukuda et al., 2000) disrupt synaptic vesicle release (Littleton et al., 2001), although the effects of this mutation on phosphoinositide and SNARE binding, as well as defects in interactions with endocytosis regulators, might also contribute to the inability of the protein to control fusion.

The effects of the C2A*-C2B* transgene on release in the null versus wild-type backgrounds argue that the mutant form of Syt1 may form multimers with native Syt1 in vivo to generate a complex with unique properties. In the absence of endogenous Syt1, the C2A*-C2B* mutant failed to rescue any aspect of evoked release (Fig. 7B). In addition, this complex would be able to dramatically enhance evoked release (Fig. 7A). We hypothesize that overexpression of C2A*-C2B* allows this fusogenic version of Syt1 to multimerize with endogenous Syt1 in both Ca\(^{2+}\)-independent and Ca\(^{2+}\)-dependent manners in vivo, as suggested by binding assays (Fig. 9). These heteromultimers exhibit characteristics of both Syt1 molecules: they are more effective in Ca\(^{2+}\)-dependent vesicle fusion, as evidenced by increased release probability (Fig. 7D) and Ca\(^{2+}\) sensitivity (Fig. 7C) while maintaining a clamping function provided by endogenous Syt1 to reduce Ca\(^{2+}\)-independent spontaneous fusion (compare Figs. 7B and 5). Although additional studies will be required to confirm that the multimeric Syt1 complex exists in vivo, our data indicate that the different modes of synaptic release, Ca\(^{2+}\)-independent spontaneous fusion and Ca\(^{2+}\)-dependent synchronous vesicle release, are likely to be regulated by multimerization of Syt1. In addition, our findings provide evidence that both C2 domains of Syt1 are uniquely required for the two forms of synaptic neurotransmitter release.

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