

# Synaptotagmin I Functions as a Calcium Sensor to Synchronize Neurotransmitter Release

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## Summary

To characterize  $\text{Ca}^{2+}$ -mediated synaptic vesicle fusion, we analyzed *Drosophila* synaptotagmin I mutants deficient in specific interactions mediated by its two  $\text{Ca}^{2+}$  binding C2 domains. In the absence of synaptotagmin I, synchronous release is abolished and a kinetically distinct delayed asynchronous release pathway is uncovered. Synapses containing only the C2A domain of synaptotagmin partially recover synchronous fusion, but have an abolished  $\text{Ca}^{2+}$  cooperativity. Mutants that disrupt  $\text{Ca}^{2+}$  sensing by the C2B domain have synchronous release with normal  $\text{Ca}^{2+}$  cooperativity, but with reduced release probability. Our data suggest the  $\text{Ca}^{2+}$  cooperativity of neurotransmitter release is likely mediated through synaptotagmin-SNARE interactions, while phospholipid binding and oligomerization trigger rapid fusion with increased release probability. These results indicate that synaptotagmin is the major  $\text{Ca}^{2+}$  sensor for evoked release and functions to trigger synchronous fusion in response to  $\text{Ca}^{2+}$ , while suppressing asynchronous release.

## Introduction

Katz and colleagues established the hypothesis that  $\text{Ca}^{2+}$  influx into the presynaptic nerve terminal triggers neurotransmitter release (Katz and Miledi, 1967). Current models for vesicle exocytosis propose that  $\text{Ca}^{2+}$  triggers fusion through activation of the SNARE complex (Söller et al., 1993; Littleton et al., 1998; Chen et al., 1999; Hu et al., 2002). The SNARE complex is formed from the interaction of the synaptic vesicle protein synaptobrevin and the plasma membrane proteins syntaxin and SNAP-25. Together these proteins assemble into a four-helix bundle (Sutton et al., 1998) that is sufficient to drive vesicle fusion in *in vitro* reconstitution experiments (Weber et al., 1998). However, vesicle fusion mediated through the assembly of reconstituted SNARE proteins is slow and  $\text{Ca}^{2+}$  independent. This sharply contrasts with synaptic transmission, where SNARE complex assembly and subsequent vesicle fusion is rapid and triggered by  $\text{Ca}^{2+}$  (Chen et al., 1999; Hu et al., 2002).

The identity of the  $\text{Ca}^{2+}$  sensor(s) that triggers vesicle fusion is still under investigation, but many studies point toward an essential function for synaptotagmin in coupling  $\text{Ca}^{2+}$  to SNARE-mediated fusion. Synaptotagmins form a large family of C2 domain-containing proteins with seven members in *Drosophila* and 19 members in

mammals (Adolfson and Littleton, 2001; Craxton, 2001). Synaptotagmin I (hereafter referred to as synaptotagmin) is the most abundant  $\text{Ca}^{2+}$  binding protein present on synaptic vesicles and accounts for 7% of total vesicle protein (Perin et al., 1990; Chapman and Jahn, 1994). Synaptotagmin contains two well-characterized  $\text{Ca}^{2+}$  binding motifs known as C2 domains. The C2 domain is an abundant motif present in over 100 human proteins (Lander et al., 2001) and was initially found to encode a  $\text{Ca}^{2+}$ -activated lipid binding domain in protein kinase C (Nishizuka, 1988). Biochemical studies have demonstrated numerous  $\text{Ca}^{2+}$ -dependent interactions mediated by synaptotagmin that suggest it may couple  $\text{Ca}^{2+}$  influx to vesicle fusion. Specifically, synaptotagmin binds the SNARE complex and individual t-SNAREs (syntaxin and SNAP-25) in a  $\text{Ca}^{2+}$ -stimulated manner (Chapman et al., 1995, 1996; Davis et al., 1999; Schiavo et al., 1997; Gerona et al., 2000; Kee and Scheller, 1996). Synaptotagmin also binds phospholipids in a  $\text{Ca}^{2+}$ -dependent manner through lipid interactions with both C2 domains (Brose et al., 1992; Chapman and Jahn, 1994; Davis et al., 1999; Südhof and Rizo, 1996; Earles et al., 2001; Fernandez et al., 2001). In addition, synaptotagmin undergoes homo-oligomerization via  $\text{Ca}^{2+}$ -dependent activation of its C2B domain (Chapman et al., 1996; Osborne et al., 1999; Fukuda et al., 2000; Sugita et al., 1996; Littleton et al., 1999).

Although synaptotagmin has been demonstrated to bind  $\text{Ca}^{2+}$  *in vitro*, it is still unknown if synaptotagmin is the major  $\text{Ca}^{2+}$  sensor for synaptic exocytosis, and if so, how it mediates  $\text{Ca}^{2+}$  activation of release. Several studies have demonstrated that synaptotagmin is required for normal synaptic transmission. Knockout mice lacking synaptotagmin have greatly reduced synchronous transmitter release following nerve stimulation (Geppert et al., 1994). Knockin mice with a mutated synaptotagmin that has a 2-fold reduction in  $\text{Ca}^{2+}$ -dependent phospholipid binding by the C2A domain display a 50% reduction in evoked release (Fernández-Chacón et al., 2001). *Drosophila* synaptotagmin (*synt*) mutants also show reduced evoked neurotransmitter release (Littleton et al., 1993b, 1994; DiAntonio and Schwarz, 1994; Broadie et al., 1994; Mackler et al., 2002). However, mutants that affect SNARE proteins also disrupt evoked release (Yoshihara et al., 1999; Schulze et al., 1995), suggesting this phenotype alone is an insufficient indicator of a specific loss of  $\text{Ca}^{2+}$  sensing versus defects in other stages of vesicle trafficking. The fourth order  $\text{Ca}^{2+}$  cooperativity of release provides the steep relationship between  $\text{Ca}^{2+}$  and vesicle fusion, and is the best indicator for a specific role in  $\text{Ca}^{2+}$  sensing (Dodge and Rahamimoff, 1967). However, alterations in the  $\text{Ca}^{2+}$  cooperativity of release have not been conclusively demonstrated for *synt* mutants. Finally, the relationship between synaptotagmin's biochemical interactions and its role in secretion is largely unknown. To address these questions and determine if synaptotagmin is the major  $\text{Ca}^{2+}$  sensor for synaptic exocytosis, we undertook an electrophysiological analysis of *Drosophila synt* mutants that disrupt distinct functions of synaptotagmin.

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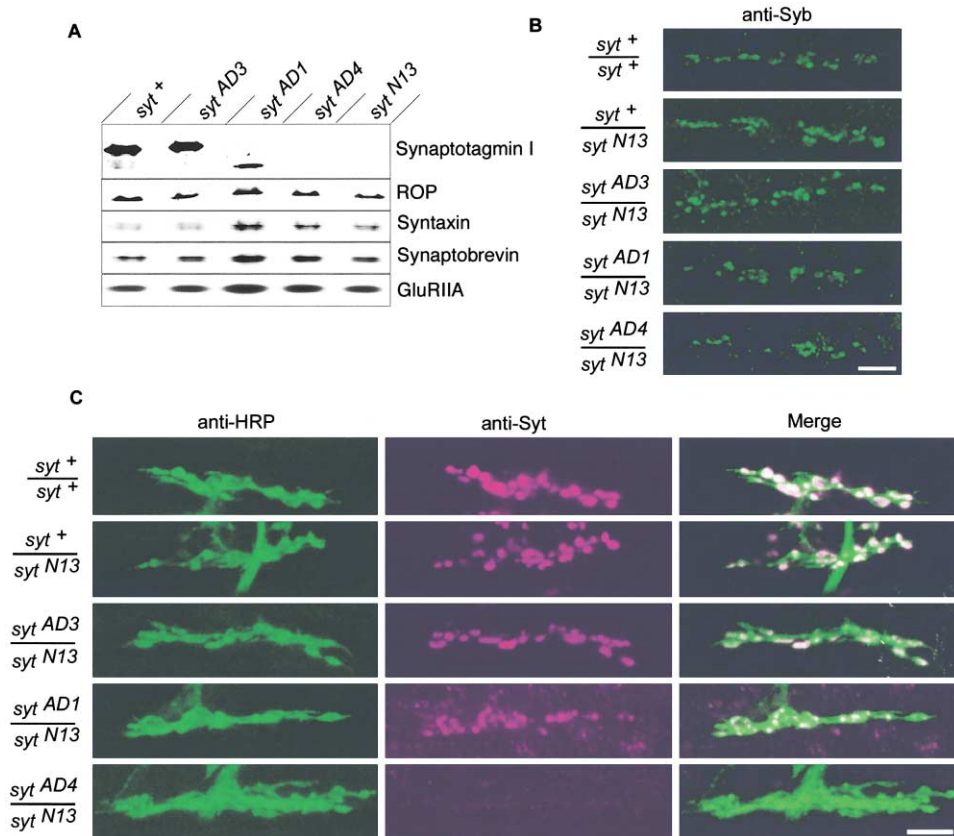


Figure 1. Immunocharacterization of syt Mutants

(A) Embryo homogenates were prepared from control syt<sup>+</sup>, syt<sup>AD3</sup>, syt<sup>AD1</sup>, syt<sup>AD4</sup>, and syt<sup>N13</sup> homozygotes in the *Mhc*<sup>1</sup> background and probed for the indicated antigens by Western analysis. Homogenates were prepared from 20 mature late stage 17 embryos in 40  $\mu$ l of sample buffer. Synaptotagmin is absent in syt<sup>AD4</sup> and syt<sup>N13</sup>, and truncated in syt<sup>AD1</sup> due to the deletion of the C2B domain. Immunoreactivity for the SNARE proteins synaptobrevin and syntaxin, as well as the glutamate receptor GluRIIA, is not reduced in the syt mutants. Precise quantification of the levels of the truncated AD1 protein is difficult, as the antibody we made against *Drosophila* synaptotagmin (DSYT2) was generated against a recombinant protein containing both the C2A and C2B domains (Littleton et al., 1993a). Given that syt<sup>AD1</sup> deletes the C2B domain, a large number of relevant epitopes are missing in the AD1 mutant protein. (B) Synaptobrevin immunoreactivity at muscle fiber 6/7 is shown in controls (syt<sup>+</sup>/syt<sup>+</sup> and syt<sup>N13</sup>/syt<sup>+</sup>) and syt mutants. The localization of synaptobrevin to synapses is unaltered in syt mutants. (C) Immunohistochemical analysis of syt mutant embryos with anti-HRP and anti-synaptotagmin. The mutated synaptotagmin proteins in syt<sup>AD3</sup> and syt<sup>AD1</sup> localize to the synapse, while syt<sup>AD4</sup> and syt<sup>N13</sup> lack synaptotagmin immunoreactivity. Anti-HRP staining demonstrates normal synaptic morphology in the absence of synaptotagmin. All images were taken with identical confocal settings for each genotype, except for anti-synaptotagmin staining in syt<sup>AD1</sup> and syt<sup>AD4</sup> mutants, where detector gain was increased. Anterior is to the left and dorsal is up in all images. The scale bar is 5  $\mu$ m.

For our studies, we analyzed three syt alleles (*AD1*, *AD3*, and *AD4*) that have been biochemically characterized and have defects in specific molecular interactions (DiAntonio and Schwarz, 1994; Littleton et al., 2001; Fukuda et al., 2000). syt<sup>AD4</sup> is a null allele caused by an early stop codon that deletes the transmembrane and cytoplasmic domains of the protein and disrupts all of synaptotagmin's known interactions (DiAntonio and Schwarz, 1994). syt<sup>AD1</sup> has a premature stop codon that deletes the C2B domain and reduces Ca<sup>2+</sup>-dependent binding of synaptotagmin to SNAREs and Ca<sup>2+</sup>-dependent oligomerization, while preserving phospholipid binding by the C2A domain (Littleton et al., 2001; Davis et al., 1999). syt<sup>AD3</sup> encodes a Y364N change in C2B that does not abolish SNARE or phospholipid binding, but instead disrupts Ca<sup>2+</sup>-dependent conformational changes in C2B that are required for oligomerization of synaptotagmin (Littleton et al., 2001; Fukuda et al., 2000). These mutations allow us to analyze and separate the role of

Ca<sup>2+</sup>-dependent phospholipid binding, Ca<sup>2+</sup>-dependent SNARE binding, and Ca<sup>2+</sup>-dependent oligomerization of synaptotagmin in vesicle fusion. We find that each of these mutants alters exocytosis in a specific fashion, indicating multiple functions for synaptotagmin.

## Results

To begin structure-function studies on synaptotagmin, we analyzed the quantity and distribution of a number of synaptic proteins in syt mutants. In the null syt<sup>AD4</sup> mutant, no synaptotagmin can be detected by Western analysis or immunocytochemistry using anti-synaptotagmin antibodies (Figures 1A and 1C). Both syt<sup>AD3</sup> and syt<sup>AD1</sup> mutants make and target the mutated synaptotagmin to synapses as assayed by immunocytochemistry and Western analysis (Figures 1A and 1C). The levels of the AD3 mutant protein are unaltered from wild-type. Immunostaining for the AD1 mutant protein is reduced,

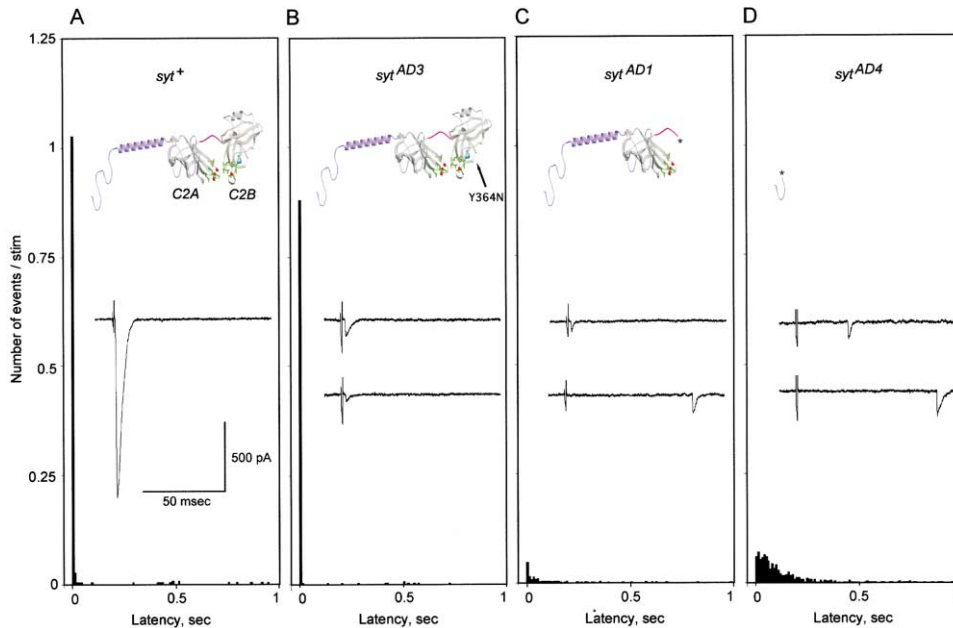


Figure 2. Latency Analysis of Evoked Neurotransmitter Release in *syt* Mutants

Genotypes are: (A) *syt*<sup>+</sup>; (B) *syt*<sup>AD3</sup>; (C) *syt*<sup>AD1</sup>; (D) *syt*<sup>AD4</sup>. The latencies of synaptic currents within 1 s following nerve stimulation were measured and plotted (100 stimuli for each cell). Results from each animal were averaged and presented as the number of events/per stimulation in 10 ms bins. The value is slightly greater than 1 in wild-type as two release events from a single stimuli were occasionally observed. The small number of random asynchronous events in wild-type are due to spontaneous neural firing originating from the intact CNS as they are large amplitude events. Traces containing spontaneous bursting activity originating from the CNS were excluded from the analysis. Spontaneous CNS activity is abolished in *syt* mutants due to the dramatic reduction in neurotransmission. Latency measurements for all alleles in 4 mM Ca<sup>2+</sup> are shown. The small number of release events in the first 10 ms bin for the *syt* null largely reflect events occurring after the first 6 ms, as normal release in the first few ms was rarely observed. A predicted model for synaptotagmin's structure (based on the crystal structure of synaptotagmin III) in each of the three mutants is indicated above. The  $\alpha$  helix represents the predicted transmembrane domain. The numbers of animals analyzed for each data point are *syt*<sup>+</sup> (4), *syt*<sup>AD3</sup> (5), *syt*<sup>AD1</sup> (10), and *syt*<sup>AD4</sup> (7).

but it is properly localized to synapses, suggesting the C2B domain is not required for synaptic targeting of synaptotagmin (Figure 1C). The reduced staining in *syt*<sup>AD1</sup> can be attributed in part to the loss of missing epitopes in the C2B domain, as the polyclonal anti-synaptotagmin antibody was made to both C2 domains (Littleton et al., 1993a). *syt* mutants did not affect neuronal pathfinding or synaptic morphology, ruling out a developmental role for synaptotagmin. In addition, Western analysis demonstrated that the mutants did not reduce the levels of the SNARE proteins syntaxin or synaptobrevin, or the postsynaptic glutamate receptor GluRIIA (Figure 1A). The distribution of synaptobrevin at synapses was also normal in *syt* mutants (Figure 1B).

To assess the physiological consequences of disrupting synaptotagmin, we measured synaptic currents arising from activation of neuromuscular junctions on muscle fiber 6 in mature *Drosophila* embryos. At this stage of development, the neuromuscular junctions on muscle fibers 6 and 7 contain approximately 8.5  $\pm$  1.7 varicosities, each containing a few active zones (Yoshihara et al., 1997). Recordings were done with *syt* alleles in *trans* to a null mutant of *syt* (*Df(2L)N13*) that specifically deletes the 5' end of the gene (Littleton et al., 1994). In control animals, nerve stimulation elicits rapid synchronous vesicle fusion that is completed in several milliseconds (Figure 2A). In *syt*<sup>AD4</sup> null mutants, synchronous release is abolished, with the appearance of resid-

ual delayed release that is not observed in controls (Figures 2D and 3A). Asynchronous release in *syt*<sup>AD4</sup> mutants following an action potential is clearly distinct from spontaneous fusion events (minis), as mini frequency is less than 0.01 Hz at these synapses (Figure 6A). Delayed release is rarely observed in wild-type, indicating that synaptotagmin I functions to suppress asynchronous release and trigger rapid and transient fusion signals that contribute to the high temporal resolution of synaptic transmission (Figure 2). The absence of asynchronous release in wild-type animals is not due to vesicle depletion following an action potential, as wild-type synapses can continue to release neurotransmitter during stimulation frequencies as high as 50 Hz (data not shown), as well as during high K<sup>+</sup>- or Ca<sup>2+</sup>-ionophore-induced release (Figures 6B and 6C). The population time constant for delayed release latencies measured over many stimulation trials in *syt*<sup>AD4</sup> was calculated from exponential fitting and is 115 ms, in dramatic contrast to the less than 6 ms time constant measured for synchronous release in wild-type. Latency histograms of delayed release in *syt*<sup>AD4</sup> (Figure 3A) reveal that asynchronous release is not detected in 1 mM Ca<sup>2+</sup>, but is robust in 2 mM Ca<sup>2+</sup>, indicating a steep Ca<sup>2+</sup> dependence. The time constant and fourth order Ca<sup>2+</sup> cooperativity of delayed release in *syt* mutants in mice (Geppert et al., 1994; Goda and Stevens, 1994) is similar to what we have measured in *Drosophila*, suggesting a con-

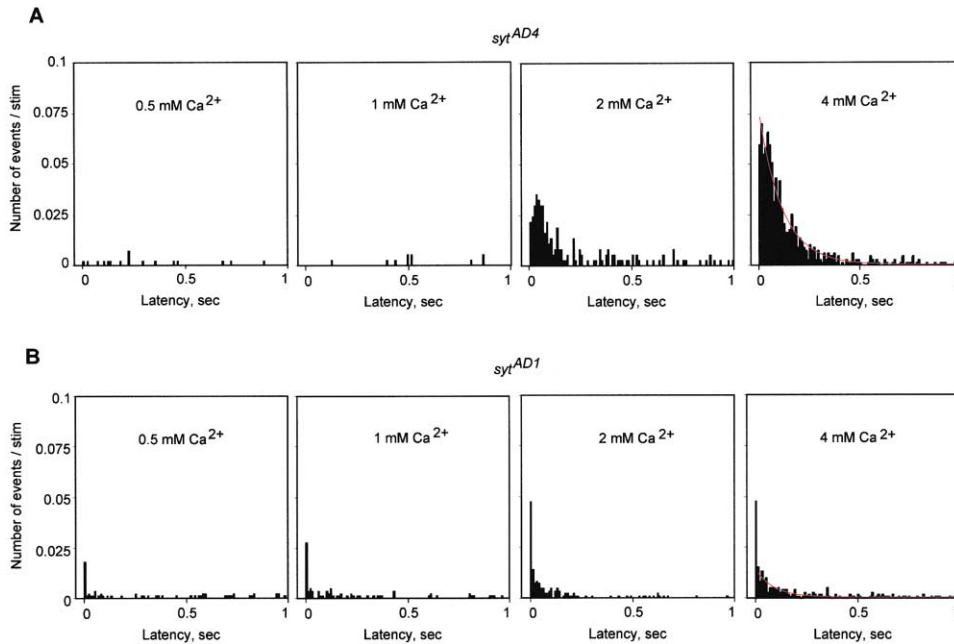


Figure 3.  $\text{Ca}^{2+}$  Dependence of Release Latency in *syt* Mutants

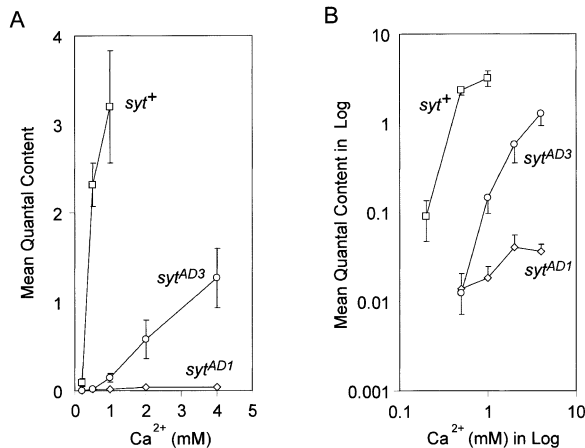
Genotypes are: (A) *syt<sup>AD4</sup>*; (B) *syt<sup>AD1</sup>*. Number of measurements in *syt<sup>AD4</sup>* mutants in 0.5 mM  $\text{Ca}^{2+}$  (4), 1 mM  $\text{Ca}^{2+}$  (4), 2 mM  $\text{Ca}^{2+}$  (4), and 4 mM  $\text{Ca}^{2+}$  (7). The red line in the last panel of (A) indicates the measured time constant for delayed release calculated from exponential fitting (115 ms). For *syt<sup>AD1</sup>* recordings, each data point is the average of nine animals. The red line in the last panel of (B) indicates the measured time constant for delayed release calculated from exponential fitting (100 ms) following removal of the first data bin containing synchronous release. Unlike *syt<sup>AD4</sup>*, *syt<sup>AD1</sup>* mutants display synchronous release with a small release probability even in 0.5 mM  $\text{Ca}^{2+}$ .

served molecular mechanism for asynchronous release. The shape of the postsynaptic response to delayed release is identical to that elicited from synchronous release in wild-type, suggesting that once the fusion pore is triggered to open, fusion kinetics are identical with or without synaptotagmin. Thus, synaptotagmin is required to rapidly trigger vesicle fusion in response to  $\text{Ca}^{2+}$  entry, while suppressing asynchronous release. This contrasts with mutations in the t-SNARE syntaxin, which abolish both synchronous and asynchronous release (Schulze et al., 1995), suggesting the SNARE complex is required for both vesicle fusion pathways. Our analysis identifies two distinct phases of neurotransmitter release at *Drosophila* synapses—a fast component completed within a few milliseconds, and a second phase of asynchronous release that has a decay time constant of 115 ms.

Given that only asynchronous fusion is present in the complete absence of synaptotagmin, we examined partial loss-of-function alleles to determine the molecular features of synaptotagmin required to suppress asynchronous fusion and trigger the rapid synchronous release observed at wild-type synapses. In contrast to the complete absence of synchronous release in *syt<sup>AD4</sup>*, *syt<sup>AD1</sup>* embryos show synchronous release, although it is greatly reduced compared to controls (Figures 2C and 3B). The synchronous component of release observed in *syt<sup>AD1</sup>* suggests that the C2A domain alone is capable of promoting rapid vesicle fusion, likely due to the preserved  $\text{Ca}^{2+}$ -dependent binding of the C2A domain to phospholipids in the *AD1* mutant (Littleton et al., 2001). A comparison of delayed release in *syt<sup>AD1</sup>* and *syt<sup>AD4</sup>*

(Figure 3) indicates that the presence of the C2A domain alone is able to suppress some, though not all, of the asynchronous release that is observed in the null mutant. Although the absolute number of asynchronous release events is reduced in *AD1*, the time constant for the remaining delayed release events is unchanged. Thus, both the synchronous and asynchronous phases of release coexist in the *AD1* mutant. Whereas asynchronous release is still present in *syt<sup>AD1</sup>*, no asynchronous release was detected in *syt<sup>AD3</sup>* (Figure 2B). Given the underlying biochemical defects in the *AD1* and *AD3* mutant proteins, the triggering of synchronous release and the suppression of asynchronous fusion events likely require synaptotagmin to engage both SNAREs and phospholipids during elevated  $\text{Ca}^{2+}$  transients, but do not require  $\text{Ca}^{2+}$ -dependent conformational changes in C2B required for oligomerization.

We next compared the properties of synchronous release remaining in *syt<sup>AD1</sup>* and *syt<sup>AD3</sup>*. As shown in Figure 4, wild-type embryonic synapses have a  $\text{Ca}^{2+}$  dependence of synchronous release with a cooperativity of 3.5 in nonsaturating  $\text{Ca}^{2+}$  ranges (between 0.2 mM  $\text{Ca}^{2+}$  and 0.5 mM  $\text{Ca}^{2+}$ ). This cooperative  $\text{Ca}^{2+}$  dependence is consistent with previous studies in mammals (Heidelberger et al., 1994; Dodge and Rahamimoff, 1967) and *Drosophila* (Littleton et al., 1994; Jan and Jan, 1976; Stewart et al., 2000) and is thought to arise from approximately 4  $\text{Ca}^{2+}$  ions that cooperatively bind to the  $\text{Ca}^{2+}$  sensor(s). In *syt<sup>AD3</sup>*,  $\text{Ca}^{2+}$  cooperativity is not altered (3.5 between 0.5 mM  $\text{Ca}^{2+}$  and 1 mM  $\text{Ca}^{2+}$ ) despite a more than 10-fold reduction in quantal content. SNARE binding is not abolished in *syt<sup>AD3</sup>* mutants, while  $\text{Ca}^{2+}$ -depen-



**Figure 4.**  $\text{Ca}^{2+}$  Sensitivity of Synchronous Release in *syt* Mutants (A) Quantal content was measured using the method of failures in control, *syt*<sup>AD3</sup>, and *syt*<sup>AD1</sup> embryos at the indicated concentrations of extracellular  $\text{Ca}^{2+}$ . Error bars are SEM. The numbers of animals averaged for each recording are *syt*<sup>+</sup> (4 in 0.2 mM  $\text{Ca}^{2+}$ , 8 in 0.5 mM  $\text{Ca}^{2+}$ , 3 in 1 mM  $\text{Ca}^{2+}$ ), *syt*<sup>AD3</sup> (3 in 0.2 mM  $\text{Ca}^{2+}$ , 6 in 0.5 mM  $\text{Ca}^{2+}$ , 7 in 1 mM  $\text{Ca}^{2+}$ , 6 in 2 mM  $\text{Ca}^{2+}$ , 6 in 4 mM  $\text{Ca}^{2+}$ ), and *syt*<sup>AD1</sup> (4 in 0.2 mM  $\text{Ca}^{2+}$ , 9 in 0.5 mM  $\text{Ca}^{2+}$ , 9 in 1 mM  $\text{Ca}^{2+}$ , 9 in 2 mM  $\text{Ca}^{2+}$ , 11 in 4 mM  $\text{Ca}^{2+}$ ). (B) Log-log plot of mean quantal content versus extracellular  $[\text{Ca}^{2+}]$ . Both wild-type and *syt*<sup>AD3</sup> animals have a cooperativity slope of 3.5, while *syt*<sup>AD1</sup> mutants have a cooperativity value of 0.77 between 0.5 mM  $\text{Ca}^{2+}$  and 2 mM  $\text{Ca}^{2+}$  and show no increase in  $\text{Ca}^{2+}$  sensitivity beyond 2 mM  $\text{Ca}^{2+}$ .

dent conformational changes required for oligomerization of synaptotagmin are severely disrupted (Littleton et al., 2001; Fukuda et al., 2000). Thus,  $\text{Ca}^{2+}$  binding by the C2B domain greatly enhances the sensitivity of vesicle fusion to  $\text{Ca}^{2+}$ , but is not required for cooperativity. In stark contrast, the cooperative  $\text{Ca}^{2+}$  dependence of synchronous release is abolished in *syt*<sup>AD1</sup> mutants (Figure 4). Mean quantal content is linearly dependent on  $\text{Ca}^{2+}$  between 0.5 mM  $\text{Ca}^{2+}$  and 2 mM  $\text{Ca}^{2+}$  (slope value of 0.77) and there is no increase in release between 2 mM and 4 mM  $\text{Ca}^{2+}$  in *syt*<sup>AD1</sup> mutants. The loss of cooperativity in *syt*<sup>AD1</sup> as opposed to *syt*<sup>AD3</sup> suggests that the  $\text{Ca}^{2+}$  cooperativity of neurotransmitter release likely arises from synaptotagmin's  $\text{Ca}^{2+}$ -dependent interaction with SNAREs. To rule out the possibility that the *syt* alleles caused dominant-negative effects on neurotransmission, we examined nerve-evoked release in 0.5 mM  $\text{Ca}^{2+}$  in heterozygotes of *syt* mutants and compared quantal contents with homozygotes containing both copies of the wild-type allele. All heterozygotes showed approximately half the quantal content of controls (*syt*<sup>+/+</sup>/*syt*<sup>+</sup>,  $2.3 \pm 0.2$  SEM,  $n = 8$ ; *syt*<sup>N13</sup>/*syt*<sup>+</sup>,  $1.0 \pm 0.3$ ,  $n = 4$ ; *syt*<sup>AD4</sup>/*syt*<sup>+</sup>,  $1.2 \pm 0.3$ ,  $n = 4$ ; *syt*<sup>AD3</sup>/*syt*<sup>+</sup>,  $0.8 \pm 0.1$ ,  $n = 5$ ; *syt*<sup>AD1</sup>/*syt*<sup>+</sup>,  $0.7 \pm 0.2$ ,  $n = 4$ ). Single factor ANOVA and Scheffe's multiple comparison tests indicated significant differences ( $p < 0.05$ ) between heterozygotes (*syt*<sup>N13</sup>/+, *syt*<sup>AD4</sup>/+, *syt*<sup>AD3</sup>/+, *syt*<sup>AD1</sup>/+) and controls (+/+), but no differences between the null alleles and *syt*<sup>AD3</sup> or *syt*<sup>AD1</sup>. These findings indicate synaptotagmin is dose dependent for neurotransmitter release and that *syt*<sup>AD3</sup> and *syt*<sup>AD1</sup> do not have dominant-negative effects.

Synaptotagmin has also been proposed to function in vesicle recycling (Zhang et al., 1994; Jorgensen et al.,

1995; Reist et al., 1998), suggesting that the reduction in quantal content in *syt* mutants could be confounded by a reduced pool size. To test this possibility, we stimulated motor nerves repetitively at 10 Hz to elevate and sustain  $\text{Ca}^{2+}$  concentrations (Yoshihara et al., 1999) to enhance release via the high-affinity  $\text{Ca}^{2+}$  sensor (Kamiya and Zucker, 1994). In *syt*<sup>AD1</sup>, *syt*<sup>AD3</sup>, and *syt*<sup>AD4</sup>, large numbers of vesicles were released during repetitive stimulation (Figures 5A and 5B). Although the total number of vesicles released was approximately 2-fold less than in wild-type, the reduction in mean quantal content in *syt*<sup>AD1</sup> (more than 100-fold) and *syt*<sup>AD3</sup> (more than 10-fold) cannot be accounted for by reductions in vesicle recycling, docking, or the generation of fusion-competent vesicles. As observed in the response to single stimuli, release during repetitive stimulation in *syt*<sup>AD4</sup> is asynchronous to nerve stimulation (Figure 5A). To further test vesicle availability, we utilized hypertonic stimulation (500 mM sucrose) to measure the readily releasable pool (Rosenmund and Stevens, 1996; Geppert et al., 1994). As shown in Figure 5C, similar numbers of vesicles were released in *syt*<sup>AD3</sup> and controls in response to hypertonic stimulation. This indicates *syt*<sup>AD3</sup> has a normal readily releasable vesicle pool size and the decrease in quantal content following nerve stimulation is caused by reduced release probability. *syt*<sup>AD1</sup> and *syt*<sup>AD4</sup> both showed a 3-fold reduction in vesicle fusion induced by hypertonic stimulation compared to wild-type. One interpretation of these results is that the readily releasable pool is slightly smaller in *syt*<sup>AD1</sup> than wild-type and may partially contribute to the reduced probability of release (though a 3-fold reduction in pool size would only be a very minor contributor to a 100-fold reduction in release probability). However, repetitive stimulation in *syt*<sup>AD1</sup> can induce the same number of fusion events as *syt*<sup>AD3</sup>, which has a normal vesicle pool size. A second interpretation we favor is that the reduced SNARE binding by synaptotagmin in *syt*<sup>AD1</sup> and *syt*<sup>AD4</sup>, as opposed to *syt*<sup>AD3</sup>, makes the fusion machinery unstable and the activation energy needed for hypertonic-induced fusion larger. This interpretation is consistent with recent studies indicating that synaptotagmin I stabilizes the fusion pore during dense core vesicle release (Wang et al., 2001) and that SNARE proteins are required for hypertonic-induced vesicle fusion (Fergestad et al., 2001).

In addition to triggering  $\text{Ca}^{2+}$ -dependent fusion, synaptotagmin has also been postulated to function as a fusion clamp, inhibiting vesicle release in the absence of  $\text{Ca}^{2+}$ . This model is supported by the increased frequency of spontaneous miniature synaptic currents (minis) in *Drosophila* hypomorphic mutants recorded late in larval development (Littleton et al., 1993b, 1994; DiAntonio and Schwarz, 1994) and by the suppression of acetylcholine release from fibroblast cells transfected with synaptotagmin I (Morimoto et al., 1995). We tested this hypothesis by examining miniature frequency in *syt*<sup>AD1</sup>, *syt*<sup>AD3</sup>, and *syt*<sup>AD4</sup>. We found no increase in mini frequency at embryonic synapses (Figure 6A), consistent with recordings from young synapses in neuronal cultures from synaptotagmin mutant mice (Geppert et al., 1994). However, as noted above, we find that synaptotagmin I suppresses delayed asynchronous release. Since delayed release is induced by residual  $\text{Ca}^{2+}$ , likely via a high-affinity  $\text{Ca}^{2+}$  sensor (Kamiya and Zucker,

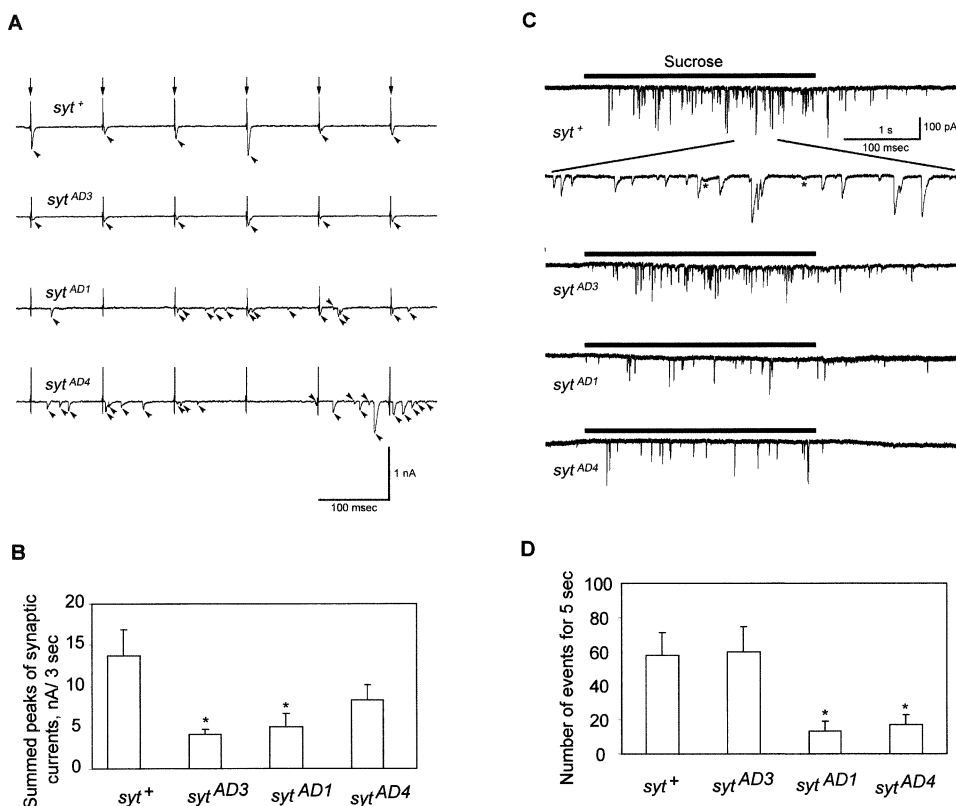
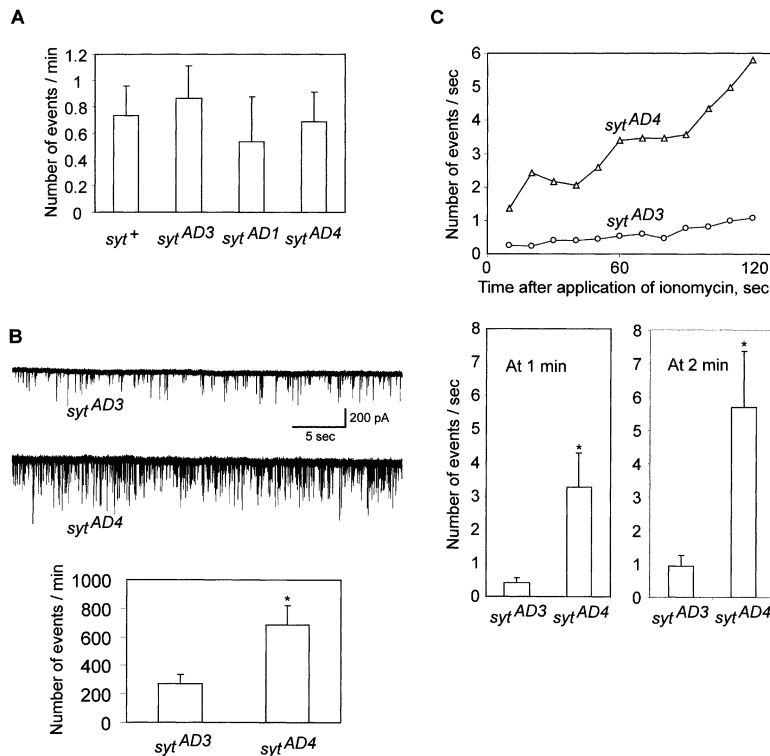


Figure 5. Release Potency Revealed by High-Frequency Stimulation and Hypertonic-Induced Fusion in *syt* Mutants

(A) Nerves were stimulated with 10 Hz pulses for 3 s and peak amplitudes of all synaptic currents during the interval were measured and summed. Panel A shows sample traces from each genotype during the onset of 10 Hz stimulation. The arrows indicate nerve stimulation artifacts and the arrowheads document release events. The quantification of release events induced by repetitive stimulation during the 3 s interval is shown in panel B. The data were analyzed by single factor ANOVA and significant differences between the groups were found ( $p < 0.01$ ). Asterisks indicate the alleles significantly different from controls using post-hoc comparisons (Dunnett's multiple comparisons test;  $p < 0.05$ ). Numbers of animals analyzed were: *syt*<sup>+</sup> (6), *syt*<sup>AD3</sup> (9), *syt*<sup>AD1</sup> (9), *syt*<sup>AD4</sup> (7). (C) Hypertonic stimulated release was achieved by puffing 500 mM sucrose onto the NMJ for 3 s in the presence of TTX (3  $\mu$ M) and 1.5 mM  $\text{Ca}^{2+}$ . Release events occurring in 5 s after the onset of stimulation were counted. Panel C shows sample traces from each genotype. Asterisks in the enlarged control trace indicate release events due to electrical coupling from adjacent muscles. Responses originating from electrically coupled muscle fibers were excluded in subsequent analysis in all genotypes. The quantification of hypertonic stimulated release is shown in panel D. The data were statistically analyzed as described above. Numbers of animals examined were: *syt*<sup>+</sup> (5), *syt*<sup>AD3</sup> (4), *syt*<sup>AD1</sup> (6), *syt*<sup>AD4</sup> (4). Error bars are SEM. The hypertonic stimulated release in *syt* null mutants is consistent with previous estimates (Fergestad et al., 2001). The calibration bar (1 s, 100 pA) applies to all low-resolution traces, while the 100 ms timescale applies only to the high-resolution trace of *syt*<sup>+</sup>. Mini amplitude, and thus postsynaptic sensitivity to released neurotransmitters, was unchanged in *syt* mutants. Mini amplitudes were: *Mhc*<sup>+</sup>,  $62.2 \pm 48.7$  (pA  $\pm$  SD;  $n = 396$  [number of measurements]); *syt*<sup>AD3</sup>,  $61.1 \pm 46.9$  ( $n = 294$ ); *syt*<sup>AD1</sup>,  $55.7 \pm 40.1$  ( $n = 218$ ); *syt*<sup>AD4</sup>,  $69.4 \pm 70.0$  ( $n = 252$ ).

1994), the lack of detectable delayed release at wild-type synapses indicates a suppression of this release mechanism by synaptotagmin I. It is known that presynaptic  $\text{Ca}^{2+}$  concentrations remain elevated for several hundred milliseconds after an action potential (Chen and Regehr, 1999), yet release events largely occur only during the early peak of the  $\text{Ca}^{2+}$  spike. The ability to restrict vesicle fusion temporally within milliseconds to the onset of a  $\text{Ca}^{2+}$  signal is an essential feature of brain function, yet the molecular machinery that mediates this critical task is unknown. Our data indicate that synaptotagmin performs this essential function by clamping asynchronous release during sustained  $\text{Ca}^{2+}$  influx. To test this hypothesis, we depolarized presynaptic terminals with high potassium ( $\text{K}^+$ ) and examined the frequency of fusion events that occur during sustained  $\text{Ca}^{2+}$  influx induced by depolarization (Yoshihara et al., 1999). We compared release properties under these

conditions in the null mutant with release in *syt*<sup>AD3</sup>, which has a normal vesicle pool size as assayed by hypertonic stimulation and shows no asynchronous fusion. As shown in Figure 6B, we observed a higher frequency of spontaneous release in *syt*<sup>AD4</sup> than in *syt*<sup>AD3</sup> in solutions containing 60 mM  $\text{K}^+$  and 0.25 mM  $\text{Ca}^{2+}$ , indicating that the lack of a synaptotagmin fusion clamp in the null mutant leads to excessive release activated through the high-affinity  $\text{Ca}^{2+}$  sensor. In addition, fusion induced by high  $\text{K}^+$  in the *syt*<sup>AD4</sup> null mutant can exceed 10 Hz and continue unabated for over an hour during continuous recordings, confirming that a large recycling vesicle pool is present in *syt* nulls and can be triggered to fuse via the asynchronous high-affinity  $\text{Ca}^{2+}$  sensor. These data exclude an essential role for synaptotagmin in synaptic vesicle endocytosis and docking. It is possible that a subpopulation of the vesicle pool at nerve terminals is not recycling properly in *syt* nulls, but our data indicate



Error bars are SEM. (C) The top panel shows the average frequencies of synaptic currents (averaged every 10 s) after the application of the  $\text{Ca}^{2+}$  ionophore ionomycin. Muscles were voltage-clamped in  $\text{Ca}^{2+}$ -free external solution with 3  $\mu\text{M}$  TTX. Five micromolar of ionomycin and 0.5 mM  $\text{Ca}^{2+}$  was then applied to the saline to trigger  $\text{Ca}^{2+}$  influx. Numbers of animals analyzed were: *syt<sup>AD3</sup>* (7), *syt<sup>AD4</sup>* (9). The bottom panel shows the average release frequencies in *syt<sup>AD3</sup>* and *syt<sup>AD4</sup>* at 1 min and 2 min after ionomycin addition. Error bars are SEM. Asterisks indicate the alleles were significantly different as analyzed by Student's *t* test ( $p < 0.05$ ). Ionophore-induced release in wild-type was not compared due to the large fraction of release directly triggered by intact synaptotagmin in these animals, making it impossible to separate release events arising from the asynchronous pathway.

that the recycling pathway remaining in *syt* mutants is capable of robustly resupplying the readily releasable vesicle pool.

To further characterize the suppression of the asynchronous release mechanism by synaptotagmin, we examined release properties in *syt<sup>AD4</sup>* and *syt<sup>AD3</sup>* in preparations where we triggered sustained elevated  $\text{Ca}^{2+}$  concentrations using the  $\text{Ca}^{2+}$  ionophore, ionomycin (Hilgenberg et al., 2002). As  $\text{Ca}^{2+}$  influx into presynaptic terminals occurs through ionomycin channels, as opposed to  $\text{Ca}^{2+}$  channels, synaptic vesicle release occurs through a general increase in nerve terminal  $\text{Ca}^{2+}$  concentrations. This provides a more appropriate experimental condition to examine the effects of sustained  $\text{Ca}^{2+}$  elevation than the high  $\text{K}^+$  experiments described above. Addition of 5  $\mu\text{M}$  ionomycin results in a large gradual increase in vesicle release secondary to the influx of  $\text{Ca}^{2+}$  through ion-permeable channels in the membrane that bypass the presynaptic  $\text{Ca}^{2+}$  channel. Under conditions of sustained  $\text{Ca}^{2+}$  influx, we observed that *syt<sup>AD4</sup>* null mutants show a  $\sim 10$ -fold higher frequency of release than in *syt<sup>AD3</sup>* mutants at 1 and 2 min following ionomycin addition (Figure 6C), suggesting that the ability of the AD3 mutant protein to function as a clamp suppresses asynchronous release responding to low  $\text{Ca}^{2+}$  concentrations via the high-affinity  $\text{Ca}^{2+}$  sensor. We conclude that synaptotagmin functions to restrict synaptic vesicle fusion temporally to the  $\text{Ca}^{2+}$

Figure 6. Suppression of Release due to Sustained Low  $\text{Ca}^{2+}$  Concentrations by Synaptotagmin

(A) Frequency of miniature synaptic currents were measured in saline containing TTX (3  $\mu\text{M}$ ) and 1.5 mM  $\text{Ca}^{2+}$  for 5 min. Numbers of animals analyzed were: *syt<sup>+</sup>* (6), *syt<sup>AD3</sup>* (6), *syt<sup>AD1</sup>* (6), *syt<sup>AD4</sup>* (7). Error bars are SEM. The previously reported increases in mini frequency in hypomorphic *Drosophila syt* mutants is likely due to developmental compensation mechanisms that occur in older animals. This conclusion is consistent with findings at third instar NMJ synapses where synaptotagmin is acutely inactivated (Marek and Davis, 2002 [this issue of *Neuron*]). (B) The frequency of synaptic currents induced by high  $\text{K}^+$  saline depolarization was measured in saline containing 3  $\mu\text{M}$  TTX, 0.25 mM  $\text{Ca}^{2+}$ , and 60 mM  $\text{K}^+$ . Release was measured for 1 min at steady state (between 3 and 4 min following  $\text{Ca}^{2+}$  application and the initial release surge). The top panel shows sample traces from each genotype. The quantification of high  $\text{K}^+$  saline-stimulated release is shown in the bottom panel. Asterisks indicate the alleles were significantly different as analyzed by Student's *t* test ( $p < 0.05$ ). Animals showing an elevated fusion rate in high  $\text{K}^+$  alone without  $\text{Ca}^{2+}$  were excluded from the analysis due to nerve damage. Numbers of animals analyzed were: *syt<sup>AD3</sup>* (5), *syt<sup>AD4</sup>* (6).

signal during an action potential and prevent further release that is asynchronous to the initial  $\text{Ca}^{2+}$  spike.

## Discussion

Our results provide convincing evidence that synaptotagmin is the major low-affinity  $\text{Ca}^{2+}$  sensor at synapses and integrates  $\text{Ca}^{2+}$  signals through several effector interactions. We summarize our results in the proposed model shown in Figure 7. The C2A domain alone can generate synchronous release with a small release probability, likely via  $\text{Ca}^{2+}$ -dependent phospholipid binding by the C2A domain. Lipid binding may allow synaptic vesicles to intimately contact the plasma membrane and facilitate SNARE complex formation (Hu et al., 2002). In addition to lipid interactions, high-affinity SNARE binding by an intact C2A-C2B domain of synaptotagmin would mediate the  $\text{Ca}^{2+}$  cooperativity of release, likely by initiating and stabilizing fusion pores formed from SNARE-synaptotagmin complexes. Cooperativity could be accounted for by multiple  $\text{Ca}^{2+}$  ions binding to one synaptotagmin monomer and triggering SNARE association (stoichiometric model, Dodge and Rahamimoff, 1967), or by the requirement of multiple synaptotagmin-SNARE interactions per fusion event (stochastic model, Dodge and Rahamimoff, 1967).  $\text{Ca}^{2+}$  binding by the C2B domain, which can promote oligomerization of synaptotagmin, dramatically increases release probability, but



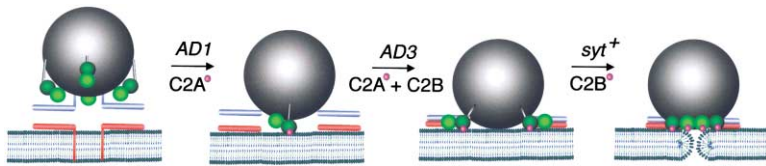


Figure 7. A Three-Stage Model for  $\text{Ca}^{2+}$ -Dependent Synaptic Vesicle Fusion Mediated by Synaptotagmin I

In the complete absence of synaptotagmin ( $\text{syt}^{\text{AD4}}$ ), no synchronous release is observed. The presence of phospholipid binding mediated by the C2A domain (preserved in  $\text{syt}^{\text{AD1}}$ ) can trigger synchronous vesicle fusion. In the

absence of high-affinity interactions with the SNARE complex, synaptotagmin's  $\text{Ca}^{2+}$ -dependent phospholipid binding ability alone has a low release probability. The high-affinity  $\text{Ca}^{2+}$ -dependent binding of synaptotagmin to SNAREs (preserved in  $\text{syt}^{\text{AD3}}$ ) would underlie the  $\text{Ca}^{2+}$  cooperativity of release and also serve to further increase release probability. Wild-type levels of evoked release would require  $\text{Ca}^{2+}$  binding to the C2B domain and subsequent oligomerization and additional lipid interactions to maximize release probability. Oligomerization may serve to focus multiple synaptotagmin-SNARE complexes at the interface of the merging lipid bilayers. Although the model is drawn stepwise for clarity, the actual sequence of molecular interactions in vivo is unknown. It is likely that they occur relatively simultaneously given the speed of vesicle fusion. The C2A domain of synaptotagmin is represented by the dark green circle, while the C2B domain is shown in light green. The v-SNARE synaptobrevin is shown in blue, while the t-SNARE syntaxin is shown in red. The transmembrane domains have been removed for clarity in all but the first panel. SNAP-25 is not shown.  $\text{Ca}^{2+}$  binding to each C2 domain is represented by the purple circles. We hypothesize that four synaptotagmin bound SNARE complexes cooperate to trigger release, but only two are shown for clarity.

likely does not contribute to  $\text{Ca}^{2+}$  cooperativity.  $\text{Ca}^{2+}$ -triggered oligomerization, which has been measured to occur on a submillisecond timescale (Davis et al., 1999), may be required to rapidly concentrate multiple synaptotagmin bound SNARE complexes into a single interface at the contact point of two lipid bilayers. The zippering together of multiple SNARE complexes, together with the lipid association mediated by the C2 domains, may be sufficient to account for the incredible speed with which fusion occurs in vivo in response to  $\text{Ca}^{2+}$ .

In addition to providing strong evidence that synaptotagmin is the  $\text{Ca}^{2+}$  sensor for the rapid phase of synaptic vesicle fusion, our data support two kinetically and mechanistically distinct phases of release, consistent with reports from mammalian CNS synapses (Goda and Stevens, 1994). Recordings from paired hippocampal neurons in culture have revealed a fast component of release with a time constant of 5–10 ms, and a second asynchronous component with a time constant of 100–200 ms (Goda and Stevens, 1994). Both of these phases of release have a cooperativity of 4 and support the two- $\text{Ca}^{2+}$  sensor model of exocytosis: a low-affinity  $\text{Ca}^{2+}$  sensor that activates the fast synchronous phase of release and a second distinct high-affinity  $\text{Ca}^{2+}$  sensor that triggers the slow asynchronous release mechanism. The similarities between mammalian CNS synapses and *Drosophila* NMJs argue that the mechanisms mediating  $\text{Ca}^{2+}$  cooperativity of release have been conserved across evolution. This work provides evidence that synaptotagmin is the only low-affinity  $\text{Ca}^{2+}$  sensor at the *Drosophila* NMJ and that it normally suppresses the second asynchronous phase of release, generating the high fidelity of normal synaptic transmission. In the complete absence of synaptotagmin ( $\text{syt}^{\text{AD4}}$ ), the fast component of release is completely abolished and the second asynchronous phase of release is fully uncovered. The cooperativity of the residual delayed release in the null mutant is unchanged, but reflects the cooperativity of the asynchronous release mechanism. Although an increased variability in release latency was previously observed (Broadie et al., 1994), the presence of two distinct release mechanisms was not previously realized and the distinction between the cooperativity of fast release versus asynchronous release was not measured. The intact cooperativity of residual release previously reported in the null mutant (Broadie et al., 1994) reflects

the cooperativity of the asynchronous release mechanism, as no synchronous release component remains in the null. In  $\text{syt}^{\text{AD1}}$ , both the synchronous and asynchronous phases coexist and can be kinetically separated, suggesting the AD1 mutant protein can trigger the fast phase of release (with a cooperativity of 1 instead of 4), but cannot fully suppress delayed release. The partial suppression of the asynchronous phase results in a decrease in the absolute number of delayed release events, but the population time constant for the remaining asynchronous events is unchanged. Both the high-affinity and low-affinity  $\text{Ca}^{2+}$  sensors that underlie these two phases of release likely impinge upon the basic SNARE fusion machinery, as mutations in syntaxin eliminate both components of release (Schulze et al., 1995).

Although the model presented in Figure 7 is consistent with our data and the previous biochemical characterization of the synaptotagmin mutant proteins used in this study, we cannot rule out that other interactions also contribute to the defects we observe in  $\text{syt}$  mutants. However, the finding that the cooperativity of neurotransmitter release ( $n$ ) is abolished in  $\text{syt}^{\text{AD1}}$  mutants (from 4 to 1) provides the best evidence to date that synaptotagmin functions as the  $\text{Ca}^{2+}$  sensor for fast exocytosis. Several other manipulations have also been reported to mildly reduce  $n$ , but none have been shown to have as severe an effect on  $\text{Ca}^{2+}$  cooperativity as  $\text{syt}$  mutants. *Drosophila dunce* mutants elevate cAMP levels and show a mild reduction in  $n$  to 2.4 (Zhong and Wu, 1991). It is possible that PKA phosphorylation patterns are altered in the *dunce* mutant and directly affect synaptotagmin-SNARE interactions. Alternatively, cAMP-dependent facilitation, which is also mediated by  $\text{Ca}^{2+}$ , may alter additional  $\text{Ca}^{2+}$  effectors of release (Yoshihara et al., 2000). Experimental decreases in the levels of the v-SNARE synaptobrevin and the t-SNARE syntaxin also cause a slight reduction in the  $\text{Ca}^{2+}$  cooperativity of release to 2.6 (Stewart et al., 2000). The reduction in SNARE proteins may cause abnormal cooperation between SNAREs and synaptotagmin during  $\text{Ca}^{2+}$  influx, thus leading to subtle reductions in cooperativity.

Given that synaptotagmin has been shown to stabilize the fusion pore during dense core vesicle fusion (Wang et al., 2001), we propose that synaptotagmin-SNARE interactions rapidly trigger the opening and stabilization



of the fusion pore, while preventing fusion pore openings induced by lower  $\text{Ca}^{2+}$  concentration via the high-affinity sensor. A role for synaptotagmin-SNARE interactions in vesicle fusion is supported by voltammetry measurements from cracked PC12 cells using dominant-negative synaptotagmin probes that prevent synaptotagmin-SNARE binding (Earles et al., 2001). In addition, the  $\text{Ca}^{2+}$ -dependent interaction of synaptotagmin with SNAP-25 has been shown to be essential for the triggering of vesicle fusion in PC12 cells (Zhang et al., 2002). Recent work on *Drosophila* synaptotagmin transgenic strains harboring mutants that alter the  $\text{Ca}^{2+}$  binding properties of the C2A and C2B domains have been reported. Disruptions of C2A  $\text{Ca}^{2+}$  binding cause a 50% decrease in evoked release from heat shock-induced mutant transgenics, but do not result in a null phenotype (Robinson et al., 2002). However, the mutant transgenes contain intact C2B  $\text{Ca}^{2+}$  binding ligands, suggesting C2B phospholipid binding may substitute for defects in C2A. Fernández-Chacón et al. (2002) showed that C2A  $\text{Ca}^{2+}$  ligand mutations engineered into the intact C2A-C2B recombinant protein do not cause defective  $\text{Ca}^{2+}$ -dependent phospholipid binding of the complete protein, confirming functional redundancy for lipid binding between the two C2 domains. Similar redundant lipid binding activities in synaptotagmin have been previously reported (Earles et al., 2001). Mutations in C2A that prevent  $\text{Ca}^{2+}$ -dependent phospholipid binding by the intact C2A-C2B domain cause a 50% decrease in release probability, confirming an important role for C2A in lipid binding (Fernández-Chacón et al., 2001). Robinson et al. (2002) also demonstrate that synaptotagmin IV, which has defective C2A phospholipid binding, can functionally substitute for synaptotagmin I. Interestingly, synaptotagmin IV maintains normal  $\text{Ca}^{2+}$ -dependent interactions with the t-SNARE syntaxin (Littleton et al., 1999), suggesting  $\text{Ca}^{2+}$ -dependent SNARE interactions may be important for synaptotagmin IV's ability to substitute for synaptotagmin I. Disruptions of the  $\text{Ca}^{2+}$  binding properties of the C2B domain result in a more severe defect in synaptic transmission, but with phenotypes that are more severe than the null mutant, indicating these transgenes function as dominant-negative inhibitors of vesicle fusion (Mackler et al., 2002). Although it is still unclear exactly how these dominant-negative phenotypes are manifested biochemically, TEM studies indicate an abundance of docked vesicles in these mutants, suggesting that  $\text{Ca}^{2+}$  binding to the C2B domain has a post-docking role in release. Although both our results and those of Mackler et al. (2002) support an essential post-docking role for synaptotagmin as the fast  $\text{Ca}^{2+}$  sensor, we cannot rule out that there may be subtle defects in endocytosis and vesicle docking. TEM analysis has revealed an overall decrease in the absolute numbers of synaptic vesicles in nerve terminals of null mutants, suggesting synaptotagmin may be required to maintain a completely wild-type pool of recycling vesicles (Reist et al., 1998). However, the TEM phenotype was similar between  $\text{sy}^{\text{AD3}}$  and null mutants. Given the striking differences in physiology between the null and  $\text{sy}^{\text{AD3}}$ , the TEM phenotype might reflect a general decrease in overall nerve terminal function that leads to less recycling, but that does not reflect a specific role for synaptotagmin in the process. Indeed,

our physiological measurements to explore the asynchronous release component remaining in the  $\text{sy}^{\text{AD3}}$  nulls suggests that robust docking and endocytosis mechanisms still exist in the absence of synaptotagmin. Release via the high-affinity  $\text{Ca}^{2+}$  sensor is robust in  $\text{sy}^{\text{AD3}}$  nulls under conditions of sustained elevated  $\text{Ca}^{2+}$  levels and can continue at high frequencies for hours. This indicates the pool of vesicles that are cycling in the  $\text{sy}^{\text{AD3}}$  null can undergo endocytosis, docking, etc. at high frequency and high fidelity, and does not support the conclusion that this population of vesicles has major defects in docking or endocytosis.

In addition to a role for synaptotagmin in triggering fast vesicle fusion, we also observe that synaptotagmin suppresses delayed release during sustained  $\text{Ca}^{2+}$  elevation. We hypothesize that the positive role of synaptotagmin as the  $\text{Ca}^{2+}$  sensor promoting the fast release phase is mechanistically distinct from its role in clamping asynchronous fusion. The best evidence for this model is that the  $\text{AD3}$  mutant has a completely intact suppression of delayed release, yet is still defective in synaptotagmin's positive role in promoting fusion.  $\text{sy}^{\text{AD3}}$  mutants also have less release evoked by salines containing high  $\text{K}^+$  or  $\text{Ca}^{2+}$  ionophores than the null mutant  $\text{sy}^{\text{AD4}}$ . The data indicate that in  $\text{sy}^{\text{AD4}}$ , where there is no promoting or clamping function of synaptotagmin remaining, there is increased release due to the unrestricted activity of the high-affinity  $\text{Ca}^{2+}$  sensor. In  $\text{sy}^{\text{AD3}}$ , the suppression of asynchronous release is still intact and thus there is less release promoted through the high-affinity sensor than in  $\text{sy}^{\text{AD4}}$ . The remaining release events in  $\text{sy}^{\text{AD3}}$  can be attributed to synaptotagmin's triggering of synchronous fusion, which is greatly reduced in this mutant. Our data do not allow us to differentiate whether synaptotagmin's suppression of asynchronous release is a  $\text{Ca}^{2+}$ -dependent or -independent function. Both asynchronous release and facilitation are postulated to be mediated by the high-affinity  $\text{Ca}^{2+}$  sensor as they share a similar  $\text{Ca}^{2+}$  dependence (Kamiya and Zucker, 1994). The identity of the high-affinity  $\text{Ca}^{2+}$  sensor responsible for delayed release is unknown, but may be another member of the large synaptotagmin family or a distinct class of  $\text{Ca}^{2+}$  binding proteins. Though facilitation is important for short-term synaptic plasticity, asynchronous release is extremely deleterious for normal synaptic transmission. The suppression of asynchronous release by synaptotagmin is critical for providing the temporal resolution of synaptic transmission. These properties make synaptotagmin I an efficient  $\text{Ca}^{2+}$  sensor that generates the high signal-to-noise ratio of neurotransmitter release that is required for brain function.

## Experimental Procedures

### Electrophysiological Analysis

Synaptic currents were recorded with the patch clamp technique in the whole-cell configuration from *Drosophila melanogaster* embryonic muscle fibers 6 in segments A2–A5 that were maintained at a holding potential of  $-60$  mV. Embryos were aged 21–24 hr after fertilization and recorded in HL3.1 saline (Y. Feng et al., 2000, Soc. Neurosci., abstract) (NaCl, 70 mM; KCl, 5;  $\text{MgCl}_2$ , 5.5;  $\text{NaHCO}_3$ , 10; Treharose, 5; Sucrose, 115; HEPES-NaOH, 5; pH 7.2) as described (Yoshihara et al., 2000) using an Axopatch 200B amplifier (Axon Instrument) at  $23^\circ\text{C}$ – $24^\circ\text{C}$ . External salines with various concentra-

tions of  $\text{Ca}^{2+}$  were prepared by replacing  $\text{MgCl}_2$  with  $\text{CaCl}_2$ . High  $\text{K}^+$  salines were prepared by replacing  $\text{NaCl}$  with  $\text{KCl}$  at the indicated concentrations. The internal solution in patch pipettes contained (in mM): CsCl, 158; ATP, 2; EGTA, 5; HEPES-NaOH, 10, pH 7.1. Late-stage *Drosophila* embryos had robust muscle contractions that make patch clamp recordings difficult. The *syt* alleles and the wild-type control were examined in combination with a muscle-specific myosin heavy chain null mutant to inhibit muscle contraction induced by  $\text{Ca}^{2+}$  influx into muscle cells, facilitating stable recordings (Yoshihara et al., 2000). The *Mhc*<sup>1</sup> mutant had no observed effect on synapse formation, neurotransmitter release, or postsynaptic glutamate receptor clustering as previously shown (Yoshihara et al., 2000). Motor nerves were positioned in a suction electrode at their site of emergence from the CNS for nerve stimulation. Before recording, embryos were treated for 1 min with 0.4 mg/ml collagenase (type IV; Sigma, St. Louis, Missouri) in 0.1 mM  $\text{Ca}^{2+}$  saline. For nerve stimulation, 2–5  $\mu\text{A}$  of positive current was passed for 1 ms through a suction electrode, which contained HL3.1 saline and was tightly attached to the nerve. Stimulation efficiency was confirmed by repetitive stimulation in 4 mM  $\text{Ca}^{2+}$  at the end of recording to monitor vesicle fusion. Mean quantal content was determined by using failure rate analysis with nerve stimulation every 3 s (Yoshihara et al., 2000). Quantal content was calculated using the following equation:  $m = \ln(N/n_0)$ , where  $n_0$  is the number of failures and  $N$  is the total number of stimuli. One hundred responses were collected and used to calculate mean quantal content. The absence of a synaptic current in 6 ms from onset of stimulation was counted as a failure. A 6 ms cutoff value was used to avoid counting delayed release, as 97% of synchronous releases following stimulation occurred within 6 ms in wild-type preparations at 0.5 mM  $\text{Ca}^{2+}$ . StatView 5.0.1 (SAS Institute Inc., North Carolina) was used for statistical analysis. Ionomycin (Sigma) was dissolved in ethanol at 5 mM and used at 5  $\mu\text{M}$  in HL3.1 saline. Tetrodotoxin (TTX, Sigma) was dissolved in distilled water at 3 mM and used at 3  $\mu\text{M}$  in HL3.1 saline.

#### Western Analysis and Immunostaining

Twenty embryos of the indicated genotype were homogenized in 40  $\mu\text{l}$  of SDS sample buffer on ice. The samples were briefly centrifuged to pellet cuticle and boiled for five minutes. Fifteen microliters of the supernatant was loaded onto 10% SDS-PAGE gels and separated at 18 mA per gel. The gels were immunoblotted with anti-DSYT2 (1:1000; Littleton et al., 1993a), anti-synaptobrevin (1:1000; van de Goor et al., 1995), anti-syntaxin 8C3 (1:1000), anti-ROP (1:1000; Harrison et al., 1994), and anti-GluRIIA 8B4D2 (1:200). Immunoreactive bands were visualized using ECL (Pierce). Immunostaining on filleted embryos was performed as previously described (Yoshihara et al., 1997). FITC-conjugated IgG against HRP, which labels neuronal cell membranes, was purchased from Cappel and used at 1:1000. DSYT2 was used at 1:1000 and anti-synaptobrevin at 1:500. Immunoreactive proteins were visualized on a Zeiss Pascal Confocal using fluorescent secondary antibodies (Molecular Probes, Chemicon).

#### Fly Stocks

All *syt* alleles were analyzed in combination with *syt*<sup>N13</sup>, a small deficiency removing the 5' end of *syt* (Littleton et al., 1994). Chromosomes containing *syt*<sup>AD1</sup>, *syt*<sup>AD3</sup>, *syt*<sup>AD4</sup>, or *syt*<sup>N13</sup> were recombined with a chromosome containing the *Mhc*<sup>1</sup> mutant to generate double mutant chromosomes. Double mutants were balanced with a CyO chromosome containing GFP driven with an actin promoter that allowed unambiguous identification of mutant embryos. *syt*<sup>+</sup> controls containing *Mhc*<sup>1</sup> were examined as homozygotes after identification using a balancer chromosome with the dominant *yellow*<sup>+</sup> marker (Yoshihara et al., 2000).

#### Acknowledgments

We thank Tsuyoshi Miyakawa for helpful advice about statistical analysis, Enrico Montana and Jeffrey Cottrell for assistance with computer software, Regis Kelly and Gerry Rubin for antibodies, and Bill Adolfsen, Guosong Liu, Ed Chapman, and Morgan Sheng for helpful discussions about the manuscript. The 8C3 antibody developed by Seymour Benzer and the 8B4D2 antibody developed by

Christopher Schuster and Corey Goodman were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, Iowa 52242. This work was supported by grants from the NIH (NS40296), the Human Frontiers Science Program Organization, and the Searle Scholars Program. J.T.L. is an Alfred P. Sloan Research Fellow.

Received: June 6, 2002

Revised: October 18, 2002

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