

# Augmenting neurotransmitter release by enhancing the apparent $\text{Ca}^{2+}$ affinity of synaptotagmin 1

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**Synaptotagmin 1 likely acts as a  $\text{Ca}^{2+}$  sensor in neurotransmitter release by  $\text{Ca}^{2+}$ -binding to its two C2 domains. This notion was strongly supported by the observation that a mutation in the C2A domain causes parallel decreases in the apparent  $\text{Ca}^{2+}$  affinity of synaptotagmin 1 and in the  $\text{Ca}^{2+}$  sensitivity of release. However, this study was based on a single loss-of-function mutation. We now show that tryptophan substitutions in the synaptotagmin 1 C2 domains act as gain-of-function mutations to increase the apparent  $\text{Ca}^{2+}$  affinity of synaptotagmin 1. The same substitutions, when introduced into synaptotagmin 1 expressed in neurons, enhance the  $\text{Ca}^{2+}$  sensitivity of release. Mutations in the two C2 domains lead to comparable and additive effects in release. Our results thus show that the apparent  $\text{Ca}^{2+}$  sensitivity of release is dictated by the apparent  $\text{Ca}^{2+}$  affinity of synaptotagmin 1 in both directions, and that  $\text{Ca}^{2+}$  binding to both C2 domains contributes to  $\text{Ca}^{2+}$  triggering of release.**

$\text{Ca}^{2+}$  sensor | exocytosis | gain-of-function | hippocampus | synaptic transmission

**C** $\text{a}^{2+}$ -evoked neurotransmitter release is a key process in interneuronal communication. The synaptic vesicle protein synaptotagmin 1 is believed to act as a  $\text{Ca}^{2+}$  sensor in the major, synchronous component of release (reviewed in refs 1 and 2). Most of the cytoplasmic region of synaptotagmin 1 is formed by two C2 domains (C2A and C2B) that share a similar  $\beta$ -sandwich structure (3, 4). The C2A and C2B domains bind three and two  $\text{Ca}^{2+}$  ions, respectively, through loops emerging at the top of the  $\beta$ -sandwich (4–6). Both synaptotagmin 1 C2 domains interact in a  $\text{Ca}^{2+}$ -dependent manner with negatively charged phospholipids (4, 7, 8), which is the most general property of C2 domains (9). In addition, the synaptotagmin 1 C2 domains exhibit a number of protein–protein interactions *in vitro*. Synaptotagmin 1 C2 domains interact with, among others, SNARE proteins (10–18), which are critical components of the membrane fusion machinery, and with synaptotagmin 1 itself (19–22).

Perhaps the most compelling evidence supporting the proposed role of synaptotagmin 1 as the major  $\text{Ca}^{2+}$  sensor that triggers synchronous neurotransmitter release was provided by the observation that a mutation in a  $\text{Ca}^{2+}$ -binding loop of the C2A domain (R233Q) causes a parallel decrease in the apparent  $\text{Ca}^{2+}$  affinity of synaptotagmin 1 in the presence of phospholipids *in vitro* and in the  $\text{Ca}^{2+}$  sensitivity of release *in vivo* (23). However, rescue experiments in *Drosophila* have led to the proposal that synaptotagmin 1 promotes release independently of the C2A domain (24). In addition, loss-of-function studies have inherent limitations because it is often unclear whether an impairment induced by a mutation is specific or results from indirect effects. To address this issue, we studied gain-of-function mutations in synaptotagmin 1 that increase its apparent  $\text{Ca}^{2+}$  affinity. We investigated a set of synaptotagmin 1 mutants in which hydrophobic residues in the  $\text{Ca}^{2+}$ -binding loops of the C2A domain, the C2B domain, or both domains were replaced with tryptophan residues. We found that tryptophan mutations in both C2 domains indeed increased the apparent  $\text{Ca}^{2+}$  affinity

of synaptotagmin 1 in the presence of phospholipids *in vitro* and led to approximately parallel increases in the  $\text{Ca}^{2+}$  sensitivity of release *in vivo*. Hence, these constitute true gain-of-function mutations for synaptotagmin 1. Together with the previous data obtained with the R233Q mutant, our results demonstrate that synaptotagmin 1 acts as a major  $\text{Ca}^{2+}$  sensor in synchronous neurotransmitter release, and that both of its C2 domains participate in  $\text{Ca}^{2+}$  sensing. In addition, our data provide further support for the notion that  $\text{Ca}^{2+}$ -dependent phospholipid binding is a primary determinant of this function.

## Methods

**Centrifugation Phospholipid-Binding Assay.** Phospholipid-binding assays were done essentially as described (18, 25) by using soluble GST-fusion proteins and heavy liposome reconstituted by synaptic vesicle lipid [composition by weight 41% phosphatidylcholine, 32% phosphatidylethanolamine, 12% phosphatidylserine, 5% phosphatidylinositol, and 10% cholesterol (26)]. All binding assays were done in a HEPES buffer (50 mM HEPES, pH 6.8/100 mM NaCl/4 mM EGTA/2 mM  $\text{MgCl}_2$ ), and free  $\text{Ca}^{2+}$  concentrations were calculated with EQCAL software (Biosoft, Cambridge, U.K.).

**Site-Directed Mutagenesis and Viral Preparation.** Mutations (C2A<sup>3W</sup>, M173W, F231W, F234W; C2B<sup>3W</sup>, V304W, Y364W, I367W) were introduced into rat synaptotagmin 1 by using standard procedures. Semliki forest virus (SFV) production was carried out as described in ref. 27. Briefly, linearized SFV plasmids were transcribed *in vitro*, and the resulting RNAs were transfected into BHK21 cells by electroporation. One day after transfection, cell culture media containing inactive virus were collected and frozen in aliquots.

**Cell Culture and Transfection.** Autaptic hippocampal cultures of neonatal synaptotagmin 1-deficient mice were prepared as described in refs. 28 and 29. Experiments were performed on neurons 10–16 days *in vitro* and 15–22 h after transfection. Only islands containing single neurons were examined. Successfully transfected neurons were detected by development of GFP-based fluorescence, resulting from internal ribosome entry site (IRES)-driven expression of the GFP. Although the viral over-

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Abbreviations: EPSC, excitatory postsynaptic current; mEPSC, miniature EPSC; PIP, phosphatidylinositol 4-phosphate;  $\text{PIP}_2$ , phosphatidylinositol 4,5-bisphosphate; RRP, readily releasable vesicle pool.

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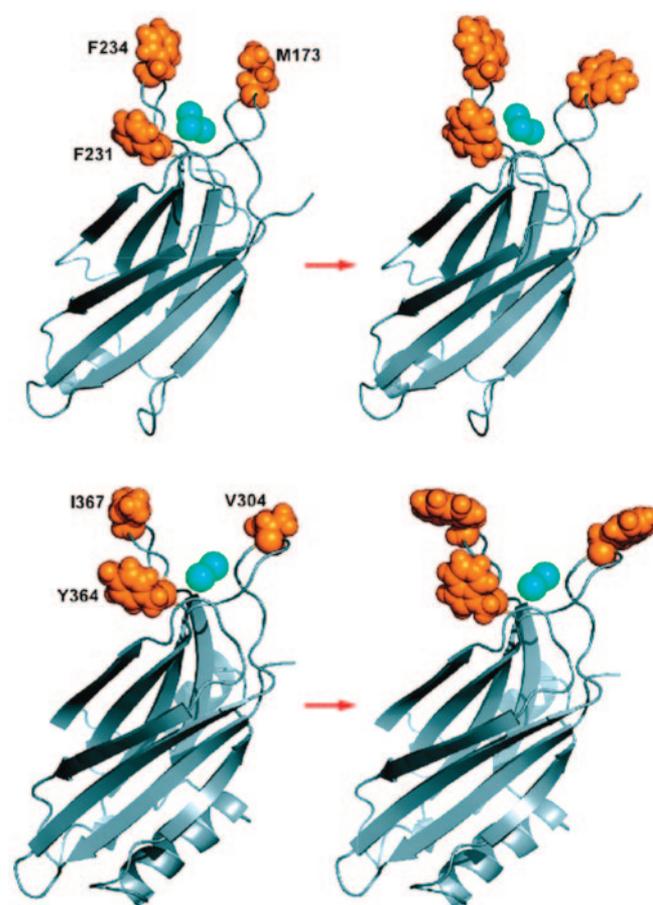
expression could, in principle, impede other cellular functions, we relied on the gain-of-function to determine whether a mutant was present on synaptic vesicles. Immunoblotting showed that Semliki forest virus expression of all synaptotagmin 1 rescue constructs led to strong overexpression, yet the C2A and C2B mutants were not expressed at significantly higher levels than in the rescue experiments with the wild-type form of synaptotagmin 1 (data not shown). In addition, SytWT-IRES-GFP-overexpressing wild-type neurons did not show any noticeable shift in the apparent  $\text{Ca}^{2+}$  sensitivity of release compared with GFP-transfected neurons (data not shown), indicating that relative degrees of overexpression of synaptotagmin 1 cannot account for the observed phenotypes.

**Electrophysiology and Statistics.** Patch-pipette solutions contained 120 mM KCl, 20 mM Hepes, 1 mM EGTA, 4.6 mM  $\text{MgCl}_2$ , 4 mM  $\text{K}_2\text{ATP}$ , 15 mM creatine phosphate, and 50 units/ml creatine kinase (0.300 osM, pH 7.3). The extracellular saline solution contained 140 mM NaCl, 2.4 mM KCl, 10 mM Hepes, 10 mM glucose, 4 mM  $\text{CaCl}_2$ , and 4 mM  $\text{MgCl}_2$ , except when otherwise noted (0.305 osM, pH 7.3). All chemicals, except for 2,3-dihydroxy-6-nitro-7-sulfamoylbenzof[*l*]quinoxaline and tetrodotoxin (Tocris Cookson, Bristol, U.K.), were purchased from Sigma. All solutions were applied by using a fast flow system at room temperature (29). Cells were whole-cell voltage clamped at  $-70$  mV with an Axopatch 200B amplifier under control of the CLAMPX 8.0 program (both Axon Instruments, Union City, CA). Currents were low-pass-filtered at 1 or 5 kHz and stored at either 10 or 20 kHz. The series resistance was compensated to 70–90%. Only cells with series resistances  $<15$  M $\Omega$  were analyzed. Data were analyzed by using AXOGRAPH 4.6 and KALEIDAGRAPH 3.0. Deconvolution of evoked neurotransmitter release was performed by a deconvolution algorithm implemented in AXOGRAPH 4.6 and was provided by John Clements (University of Canberra, Australia). Statistical significance was tested by using unpaired, nonparametric Wilcoxon tests. All values are presented as the mean  $\pm$  SE.

## Results

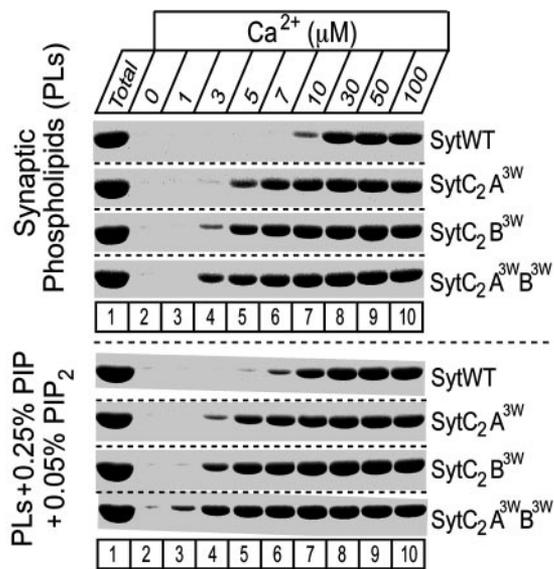
**Synaptotagmin 1 Mutants with Increased Apparent  $\text{Ca}^{2+}$  Affinity.** The gain-of-function mutants of synaptotagmin 1 were designed based on our knowledge of the mechanism of  $\text{Ca}^{2+}$ -dependent phospholipid binding to the C2A domain, which involves multiple types of interactions (30) such as: (i) electrostatic forces arising both from the drastic switch in electrostatic potential caused by  $\text{Ca}^{2+}$  binding (6, 31) and from direct interactions between basic residues around the  $\text{Ca}^{2+}$ -binding region and the negatively charged phospholipids (23, 30); (ii) partial coordination of the  $\text{Ca}^{2+}$  ions by the phospholipid head groups, because phospholipids dramatically increase the  $\text{Ca}^{2+}$  affinity (32); and (iii) hydrophobic interactions due to insertion of exposed hydrophobic residues from the  $\text{Ca}^{2+}$ -binding loops into the lipid bilayer (33–35). Although less extensively studied,  $\text{Ca}^{2+}$ -dependent phospholipid binding to the synaptotagmin 1 C2B domain likely involves the same types of interactions because its  $\text{Ca}^{2+}$ -binding loops share similar structural features with those of the C2A domain (4). Based on these considerations, increasing the exposed hydrophobic surface area of the  $\text{Ca}^{2+}$ -binding loops of the synaptotagmin 1 C2 domains may enhance phospholipid binding and increase the apparent  $\text{Ca}^{2+}$  affinity, much as the R233Q mutation indirectly decreased the apparent  $\text{Ca}^{2+}$  affinity by removing favorable electrostatic interactions with the lipids (23). Indeed, we previously showed that replacing three exposed hydrophobic residues from the  $\text{Ca}^{2+}$ -binding loops of the isolated synaptotagmin 1 C2A domain leads to a substantial increase in its apparent  $\text{Ca}^{2+}$  affinity (25).

With the goal of creating a panel of synaptotagmin 1 gain-of-function mutants, we prepared synaptotagmin 1 fragments con-



**Fig. 1.** Models of the C2A domain (Upper) and C2B domain (Lower) of synaptotagmin 1 before (Left) and after (Right) mutating exposed hydrophobic residues of the  $\text{Ca}^{2+}$ -binding loops to tryptophans. The mutated residues and the tryptophans are shown as orange space-filling models, and the  $\text{Ca}^{2+}$  ions are represented by cyan spheres. The models illustrate the increase in exposed hydrophobic surface area and the drastic change in shape that results from the mutations.

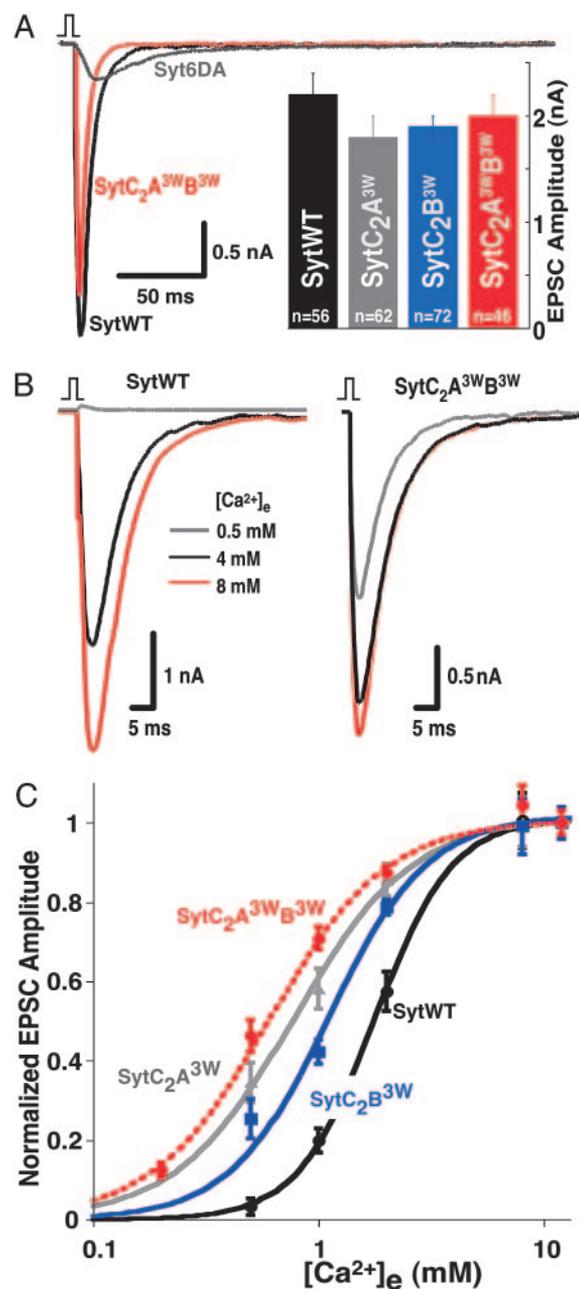
taining both C2 domains (C2AB) where the same three hydrophobic residues of the C2A domain, the three homologous residues of the C2B domain, or both domains were replaced with tryptophan residues (named SytC2A<sup>3W</sup>, SytC2B<sup>3W</sup>, and SytC2A<sup>3W</sup>B<sup>3W</sup> mutants, respectively) (see Fig. 1). We then tested  $\text{Ca}^{2+}$ -dependent phospholipid binding to the wild-type and mutant C2AB domain fragments by using a centrifugation assay (4). A lipid composition that is thought to approximate that of synaptic membranes (26, 36) was used for these experiments. As expected, the triple tryptophan substitutions in either the C2A or C2B domain substantially increased the apparent  $\text{Ca}^{2+}$  affinity of the C2AB fragment (Fig. 2). The mutations in the C2B domain had a slightly stronger effect than the mutations in the C2A domain, revealing some degree of asymmetry in the contributions of both C2 domains to the interaction. A further increase in apparent  $\text{Ca}^{2+}$  affinity was observed when both C2 domains were mutated (Fig. 2), showing that the effects of both triple mutations are at least partially additive. Because phosphoinositides have been shown to enhance lipid binding to synaptotagmin 1 (30, 37, 38), we also tested the effect of phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) on these experiments and observed an increase in the apparent  $\text{Ca}^{2+}$  affinities of all proteins (Fig. 2), but the relative effects of the mutations were similar in the presence and absence of phosphoinositides.



**Fig. 2.**  $\text{Ca}^{2+}$ -dependent phospholipid binding properties of SytWT, SytC2A<sup>3W</sup>, SytC2B<sup>3W</sup>, and SytC2A<sup>3W</sup>C2B<sup>3W</sup>. Wild-type or mutant forms of GST-synaptotagmin 1 C2AB domains were incubated with liposomes (41% phosphatidylcholine, 32% phosphatidylethanolamine, 10% phosphatidylserine, 5% phosphatidylinositol, 10% cholesterol) containing no PIP and PIP<sub>2</sub> (Upper), or 0.25% PIP + 0.05% PIP<sub>2</sub> (Lower) in the presence of free  $\text{Ca}^{2+}$  at the concentrations shown, clamped by  $\text{Ca}^{2+}/\text{Mg}^{2+}/\text{EGTA}$  buffers. Liposomes were centrifuged and washed, and bound proteins were analyzed by SDS/PAGE and Coomassie blue staining. Data shown are representative of experiments performed multiple times.

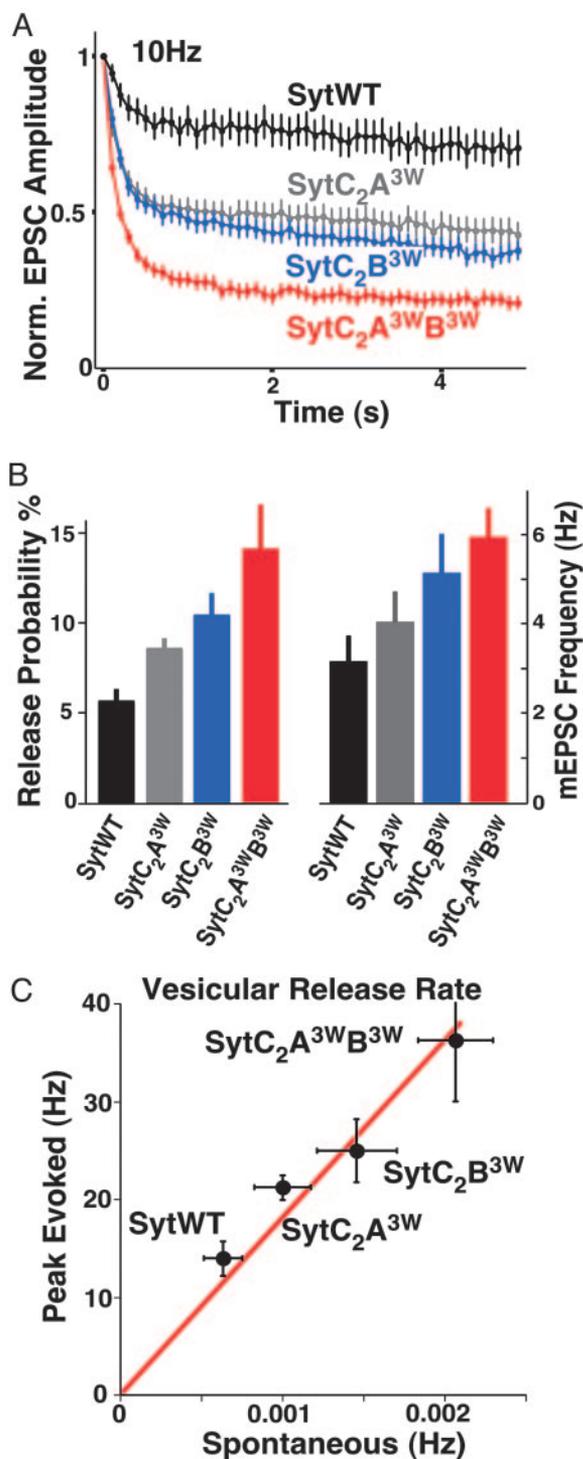
**Increased Apparent  $\text{Ca}^{2+}$  Affinity Leads to Gain-of-Function.** To test the effects of the tryptophan mutations in the synaptotagmin 1 C2 domains on neurotransmitter release, we performed rescue experiments in excitatory hippocampal neurons from synaptotagmin 1 knockout (Syt<sup>-/-</sup>) mice. Synaptotagmin 1 wild-type (SytWT) expression in Syt<sup>-/-</sup> neurons as well as rescue with the three mutant constructs led to almost full restoration of excitatory postsynaptic current (EPSC) amplitudes (ref. 28; and see Fig. 3A), confirming that all three mutants efficiently rescue fast synaptic transmission.

We examined the apparent  $\text{Ca}^{2+}$  sensitivity of triggered release upon rescue with SytWT and the tryptophan mutants by measuring EPSC amplitudes as a function of external  $\text{Ca}^{2+}$  concentration (0.2–12 mM/1 mM  $\text{Mg}^{2+}$ ; Fig. 3B and C). We found that the synaptic responses of neurons expressing any of the mutants displayed a substantial increase in apparent  $\text{Ca}^{2+}$  sensitivity compared with SytWT rescued neurons. When we lowered external  $\text{Ca}^{2+}$  to 0.5 mM, the EPSC response of the SytC2A<sup>3WB3W</sup> was still  $51 \pm 4\%$  of the control conditions, whereas SytWT responses were barely detectable ( $5 \pm 2\%$  of control) (Fig. 3B). Standard Hill equation fit of the dose-response function showed, in addition to a 3-fold increase in the apparent affinity for external  $\text{Ca}^{2+}$  ( $K_d$ , SytWT  $1.8 \pm 0.1$  mM vs. SytC2A<sup>3WB3W</sup>  $0.6 \pm 0.04$  mM), also a substantial decrease in cooperativity (Hill coefficient for SytWT =  $2.5 \pm 0.3$  vs. Hill coefficient for SytC2A<sup>3WB3W</sup> =  $1.6 \pm 0.3$ ). Interestingly, among the two individual C2<sup>3W</sup> mutants, the SytC2A<sup>3W</sup> was closer to the double mutant, whereas the SytC2B<sup>3W</sup> mutant was between the double mutant and the SytWT (SytC2A<sup>3W</sup> Hill coefficient =  $1.7 \pm 0.3$ ,  $K_d = 0.77 \pm 0.07$  mM; SytC2B<sup>3W</sup> Hill coefficient =  $2.0 \pm 0.2$ ,  $K_d = 1.11 \pm 0.07$  mM). Nevertheless, our results clearly demonstrate that strengthening the apparent  $\text{Ca}^{2+}$  affinity of either the C2A or the C2B domain of synaptotagmin 1 by increasing the hydrophobic surface area of the  $\text{Ca}^{2+}$ -binding loops of the C2 domains enhances the apparent  $\text{Ca}^{2+}$  sensitivity



**Fig. 3.** Enhanced apparent  $\text{Ca}^{2+}$  sensitivity of glutamate release in hippocampal synapses expressing SytC2<sup>3W</sup> mutants. (A) Exemplary evoked synaptic currents from hippocampal Syt<sup>-/-</sup> neurons rescued by overexpression of SytWT (black), SytC2A<sup>3WB3W</sup> mutant (red), and negative control mutant SytC2A3DAC2B3DA (gray), which contains 6 C2A/B top loop aspartate-to-alanine substitutions that eliminate  $\text{Ca}^{2+}$ -dependent phospholipid binding and rescue activity (28). (Inset) Bar plot shows mean EPSC amplitudes of SytWT (black), SytC2A<sup>3W</sup> (gray), SytC2B<sup>3W</sup> (blue), and SytC2A<sup>3WB3W</sup> (red) rescues. Number of cells is indicated within each bar. (B) Exemplary EPSCs from SytWT rescues (Left) and SytC2A<sup>3WB3W</sup> rescues (Right) at 0.5 mM (gray trace), 4 mM (black), and 8 mM (red) external  $\text{Ca}^{2+}$ . (C) Mean apparent  $\text{Ca}^{2+}$  sensitivity of EPSC amplitudes in SytWT and the three SytC2<sup>3W</sup> rescues. EPSC amplitudes measured at 0.2–12 mM external  $\text{Ca}^{2+}$  (with 1 mM  $\text{Mg}^{2+}$  held constant) were normalized to the response evoked at the highest  $\text{Ca}^{2+}$  concentration, and the data were fitted with a Hill function. SytC2A<sup>3WB3W</sup>,  $n = 12$ ; SytWT,  $n = 18$ ; SytC2A<sup>3W</sup>,  $n = 10$ ; and SytC2B<sup>3W</sup>,  $n = 12$ .

of the synaptic response. Furthermore, the decreased cooperativity in the tryptophan mutants suggests that the interaction with the membrane became less  $\text{Ca}^{2+}$ -dependent.



**Fig. 4.** Increased vesicular release probability in the gain-of-function synaptotagmin 1 mutants. (A) Plot of mean normalized EPSC amplitudes of SytWT and all three SytC<sup>3W</sup> mutants rescues during a high stimulation 10-Hz action potential train (5-s duration). (B) Bar plot of the vesicular release probability (Left) and miniature EPSC (mEPSC) frequency (Right). (C) Correlation of vesicular release rates during spontaneous release and at the peak of the evoked response in SytWT and SytC<sup>3W</sup>, SytC<sup>3W</sup>, and SytC<sup>3W</sup>B<sup>3W</sup> mutant rescues. The linear fit corresponds to a constant increase in release rate by the Ca<sup>2+</sup>-triggering event by  $\approx 18,200$ -fold.

To show that the increased Ca<sup>2+</sup> sensitivity of release in the synaptotagmin 1 tryptophan mutants is linked to an increase in the efficiency of triggered release, we studied short-term plas-

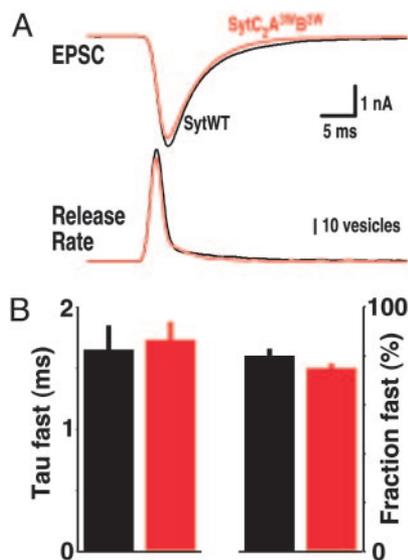
ticity in neurons rescued with the synaptotagmin 1 mutants. In general, low release probability synapses display facilitation, whereas more efficient synapses show depression (39). We monitored EPSC amplitudes during 10-Hz action potential (AP) trains. Whereas neurons rescued with SytWT showed moderate depression of EPSC amplitudes, neurons rescued by Syt 3W/6W mutants displayed graded increases in synaptic depression (SytC<sup>3W</sup>B<sup>3W</sup> > SytC<sup>3W</sup> > SytC<sup>3W</sup>A > SytWT; Fig. 4A) indicative of a strongly enhanced release probability in these mutants.

Next, we measured vesicular release probability by computing the ratio of the EPSC charge to the readily releasable vesicle pool (RRP) charge. The RRP itself can be readily determined by short, pulsed application of hypertonic solution to the entire synapse population (40). In addition, this method of evoking vesicle release has the important advantage of not depending on elevation of intracellular Ca<sup>2+</sup>. We found that, compared with the wild-type synaptotagmin 1 rescues, the neurons rescued with SytC<sup>3W</sup> mutants had moderate but significant reductions of the RRP (SytWT,  $0.50 \pm 0.07$  nC,  $n = 44$ ; SytC<sup>3W</sup>,  $0.40 \pm 0.06$  nC,  $n = 37$ ; SytC<sup>3W</sup>B<sup>3W</sup>,  $0.35 \pm 0.06$ ,  $n = 42$ ; SytC<sup>3W</sup>A<sup>3W</sup>B<sup>3W</sup>:  $0.29 \pm 0.04$  nC,  $n = 36$ ,  $P < 0.01$  for SytWT compared with SytC<sup>3W</sup>A<sup>3W</sup>B<sup>3W</sup>). The vesicular release probability increased in the order SytWT < SytC<sup>3W</sup>A < SytC<sup>3W</sup>B < SytC<sup>3W</sup>A<sup>3W</sup>B<sup>3W</sup> (Fig. 4B). Thus, the increase in the Ca<sup>2+</sup> sensitivity of release results in an increase in the efficiency of release.

**Tryptophan Mutations Increase Spontaneous Release but Do Not Affect the Time Course of Release.** We also examined the effect of the synaptotagmin 1 tryptophan mutations on spontaneous release. mEPSC amplitudes were comparable in all four groups (SytWT  $23.9 \pm 2.3$  pA,  $n = 25$ ; SytC<sup>3W</sup>  $22.3 \pm 1.1$ ,  $n = 27$ ; SytC<sup>3W</sup>B<sup>3W</sup>  $24.8 \pm 1.1$ ,  $n = 23$ ; SytC<sup>3W</sup>A<sup>3W</sup>B<sup>3W</sup>  $23.4 \pm 1.3$  pA,  $n = 21$ ). Interestingly, we found that the spontaneous release rates in the mutants increased despite their decreased RRP size (Fig. 4B), and that the spontaneous release rate per vesicle (as computed by dividing the mEPSC frequency by the number of vesicles in the RRP) increased to a similar extent as the evoked release rates (Fig. 4C).

The similar impact of the tryptophan mutations on both spontaneous and Ca<sup>2+</sup>-triggered release suggests that some of their effects may be mediated by a Ca<sup>2+</sup>-independent mechanism. Alternatively, the increase of spontaneous release may arise from a gain in sensitivity to residual intraterminal Ca<sup>2+</sup> concentrations. We tested the latter possibility by analyzing whether the increased mEPSC rate in the SytC<sup>3W</sup>B<sup>3W</sup> mutant persisted after a treatment that should lead to at least transiently to lower background intracellular Ca<sup>2+</sup> concentrations. Preincubation of neurons with EGTA/acetoxymethyl ester (50  $\mu$ M for 15 min at 37°C) immediately preceding the experiment did not reduce spontaneous mEPSC release rates in either SytWT ( $93.7 \pm 12.5\%$ ,  $n = 17$ ) or SytC<sup>3W</sup>B<sup>3W</sup> ( $81.1 \pm 16.2\%$ ,  $n = 19$ ) rescued neurons. In contrast, this treatment led in both groups to a 40–60% reduced probability of Ca<sup>2+</sup>-triggered release (data not shown), confirming the effectiveness of EGTA/acetoxymethyl ester treatment. Taken together, our data suggest that the tryptophan mutations result in corresponding increases in the probabilities of Ca<sup>2+</sup>-evoked and spontaneous release.

The available Ca<sup>2+</sup>-triggered and spontaneous vesicular release rates in this series of synaptotagmin rescues also allow us to estimate the rate increase caused by the Ca<sup>2+</sup>-triggering event (Fig. 4C). Whereas rescues with even the strongest mutant (SytC<sup>3W</sup>A<sup>3W</sup>B<sup>3W</sup>) increased release rates over the SytWT control  $\approx 3$ -fold, the maximal increase of release rate by the Ca<sup>2+</sup> trigger in neurons rescued with SytWT and all three SytC<sup>3W</sup> mutants was consistently  $\approx 18,200$ -fold. This rate increase corresponds to



**Fig. 5.** Enhanced  $\text{Ca}^{2+}$  sensitivity of triggered neurotransmitter release does not affect the time course of neurotransmitter release. (A Upper) Exemplary EPSCs from SytWT (black trace) and SytC2A<sup>3W</sup>/2B<sup>3W</sup> rescues (red trace). (A Lower) Corresponding vesicle release rate after deconvolution of EPSC with mean mEPSC time course. (B) Bar plot of mean time course (Left) and relative amplitude of the fast component of release (Right). The time courses of the decay phase were individually fitted with two exponentials to reveal the fast and slow components of vesicular release. Color code as in A. The time course of the slow component of release was not significantly changed. (SytWT,  $n = 22$ ; Syt6W,  $n = 26$ .)

a maximal energy contribution of 5.9 kcal/mol for the  $\text{Ca}^{2+}$ -triggering event.

Last, we wanted to test whether the affinity of the  $\text{Ca}^{2+}$  sensor synaptotagmin 1 affects the time course of neurotransmitter release. The vesicle release time course will, in principle, be determined by the (i) action potential induced  $\text{Ca}^{2+}$  transient, (ii) the affinity/kinetics of  $\text{Ca}^{2+}$ - $\text{Ca}^{2+}$ -receptor interaction, and (iii) the fusion rate once the  $\text{Ca}^{2+}$  trigger exerts its function. We thus tested whether a 3-fold increase in apparent  $\text{Ca}^{2+}$  sensitivity (by using the SytC2A<sup>3W</sup>/2B<sup>3W</sup> mutant) causes a change in the release time course. To analyze release time course, we deconvolved the EPSC time course in SytWT and SytC2A<sup>3W</sup>/2B<sup>3W</sup> rescues with their mEPSC time courses. Surprisingly, the release time course was not significantly changed (Fig. 5). This result suggests that the time course of release is at most poorly correlated with the intrinsic  $\text{Ca}^{2+}$  sensitivity of synaptotagmin 1 and with the  $\text{Ca}^{2+}$  sensitivity of release.

## Discussion

Previous studies of the role of synaptotagmin 1 in neurotransmitter release relied on loss-of-function mutations (e.g., see refs. 23, 24, and 41–45). Although these studies established a central role for synaptotagmin 1 in the  $\text{Ca}^{2+}$ -triggering of release, the loss-of-function approach has intrinsic limitations because it is difficult to rule out that such mutations cause indirect effects that are unrelated to the particular biochemical property that was intended to be perturbed. To address this concern, we have taken the opposite approach and used gain-of-function mutants of synaptotagmin 1, exploiting the wealth of biochemical and structural data available on its C2 domains. Together with our previous study of the R233Q mutant (23), the data obtained with the tryptophan mutants reveal a remarkable correlation between the effects of mutations in synaptotagmin 1 C2 domains on their apparent  $\text{Ca}^{2+}$  affinity *in vitro* and the effects of the same mutations on the  $\text{Ca}^{2+}$  sensitivity of neurotransmitter release *in vivo*. We established this correlation by using point mutations that involve exposed residues and that have

defined effects on a well characterized biochemical activity of the synaptotagmin 1 C2 domains and  $\text{Ca}^{2+}$ /phospholipid binding (9).

The biochemical/functional correlation revealed by our results adds to mounting evidence supporting the notion that  $\text{Ca}^{2+}$ -dependent phospholipid binding underlies the  $\text{Ca}^{2+}$  sensing function of synaptotagmin 1 in release (18, 23, 25, 46). The tryptophan mutations are expected to drastically change the shape of the  $\text{Ca}^{2+}$ -binding loops of the C2 domains (see Fig. 1). Thus, it is possible, even likely, that if the  $\text{Ca}^{2+}$ -binding loops participate in specific protein–protein interactions, such interactions may be destabilized by the tryptophan mutations. In contrast, the same mutations enhance phospholipid binding because they increase the hydrophobic surface area of the  $\text{Ca}^{2+}$ -binding loops (25, 34). We cannot exclude that the tryptophan mutations also enhance SNARE binding to synaptotagmin 1, but testing this possibility is hindered by the multitude of  $\text{Ca}^{2+}$ -dependent and -independent interactions between synaptotagmin 1 and individual SNAREs and their complexes that have been described.

We observed no apparent change in the time course of release in the strongest gain-of-function synaptotagmin 1 mutant, despite the fact that the mutant displayed an apparent 3-fold increase in  $\text{Ca}^{2+}$  affinity of release. This lack of an effect in the time course correlates with recent findings that the  $\text{Ca}^{2+}$  concentrations rise and decay very rapidly at the release site, and that the  $\text{Ca}^{2+}$  sensor equilibrates with the intraterminal  $\text{Ca}^{2+}$  on a submillisecond time scale (47).  $\text{Ca}^{2+}$  dissociates from the C2 domains of synaptotagmin 1 with a time constant of 2,000–6,000  $\text{s}^{-1}$  (48, 49), and the  $\text{Ca}^{2+}$  sensor at the calyx of held synapse is suggested to unbind  $\text{Ca}^{2+}$  with a time constant of  $\approx 3,000 \text{ s}^{-1}$  (47). An increase of affinity by slowing unbinding or increasing binding 3-fold in the SytC2A<sup>3W</sup>/2B<sup>3W</sup> mutant would be predicted to lead to a significant increase in EPSC amplitude, consistent with our observation. However, an additionally predicted decrease of 0.5–1 ms in the rise time of the EPSC (47) may be undetectable in our preparation because, in autaptic cultures, measurements of rise times are likely contaminated by the delay in release because of the variable length of the axon. Because we did not observe any changes in the EPSC decay kinetics in the neurons that were rescued with the SytC2A<sup>3W</sup>/2B<sup>3W</sup> mutant, we consider it unlikely that EPSC decay kinetics are under major influence by the  $\text{Ca}^{2+}$  affinity of synaptotagmin. It is possible that release decay kinetics are determined more by the kinetics of fusion than by the kinetics of  $\text{Ca}^{2+}$  binding to the  $\text{Ca}^{2+}$  sensor.

Another interesting observation is that spontaneous and  $\text{Ca}^{2+}$ -triggered release were equally affected by the tryptophan mutants. Although the two different modes of release vary by almost four orders of magnitude, their rates were increased to similar degrees by each individual mutant. This observation provides a strong link between the spontaneous and  $\text{Ca}^{2+}$ -evoked modes of release. It seems reasonable that the increased membrane affinity resulting from the tryptophan mutations may increase the likelihood of membrane binding at low  $\text{Ca}^{2+}$  concentrations or even in the absence of  $\text{Ca}^{2+}$ . Such increase in intrinsic membrane affinity could underlie the decrease in the  $\text{Ca}^{2+}$  cooperativity of release as well as the increase in mEPSC frequency induced by these mutations.

Finally, our data provide insight into the relative contributions of the two C2 domains of synaptotagmin 1 to release. It has been proposed that neurotransmitter release is independent of  $\text{Ca}^{2+}$  binding to the synaptotagmin 1 C2A domain (24). However, the comparable effects on release described here for the SytC2A<sup>3W</sup> and SytC2B<sup>3W</sup> mutations show that both synaptotagmin 1 C2 domains contribute to the  $\text{Ca}^{2+}$  sensitivity of release. The SytC2B<sup>3W</sup> mutation appears to increase the apparent  $\text{Ca}^{2+}$  affinity of synaptotagmin 1 to phospholipids (Fig. 2) and the probability of release (Fig. 4 A and B) more than the SytC2A<sup>3W</sup> mutation. In contrast, the SytC2A<sup>3W</sup> mutation appears to increase the  $\text{Ca}^{2+}$  sensitivity of release to a larger extent than the SytC2B<sup>3W</sup> (Fig. 3C). Although the differences between the effects of the tryptophan mutations in the

