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A Complexin/Synaptotagmin 1 Switch Controls Fast Synaptic Vesicle Exocytosis

Jiong Tang,[1,5] Anton Maximov,[1,5] Ok-Ho Shin,[1] Han Dai,[2] Josep Rizo,[2] and Thomas C. Südhof[1,3,4,*]
[1]The Center for Basic Neuroscience
[2]Department of Biochemistry and Department of Pharmacology
[3]Department of Molecular Genetics
[4]Howard Hughes Medical Institute
UT Southwestern Medical Center, Dallas, TX 75390, USA
[5]These authors contributed equally to this work.
*Contact: thomas.sudhof@utsouthwestern.edu
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SUMMARY

Ca^{2+} binding to synaptotagmin 1 triggers fast exocytosis of synaptic vesicles that have been primed for release by SNARE-complex assembly. Besides synaptotagmin 1, fast Ca^{2+}-triggered exocytosis requires complexins. Synaptotagmin 1 and complexins both bind to assembled SNARE complexes, but it is unclear how their functions are coupled. Here we propose that complexin binding activates SNARE complexes into a metastable state and that Ca^{2+} binding to synaptotagmin 1 triggers fast exocytosis by displacing complexin from metastable SNARE complexes. Specifically, we demonstrate that, biochemically, synaptotagmin 1 competes with complexin for SNARE-complex binding, thereby dislodging complexin from SNARE complexes in a Ca^{2+}-dependent manner. Physiologically, increasing the local concentration of complexin selectively impairs fast Ca^{2+}-triggered exocytosis but retains other forms of SNARE-dependent fusion. The hypothesis that Ca^{2+}-induced displacement of complexins from SNARE complexes triggers fast exocytosis accounts for the loss-of-function and gain-of-function phenotypes of complexins and provides a molecular explanation for the high speed and synchronicity of fast Ca^{2+}-triggered neurotransmitter release.

INTRODUCTION

When an action potential invades a nerve terminal, inflowing Ca^{2+} stimulates fast, submillisecond neurotransmitter release by synaptic vesicle exocytosis (Katz, 1969; Südhof, 2004). Fast synaptic exocytosis is triggered by Ca^{2+} binding to synaptotagmin 1 (Fernandez-Chacon et al., 2001) and is mediated by assembly of SNARE complexes from the SNARE proteins synaptobrevin/VAMP, syntaxin 1, and SNAP-25 (reviewed in Brunger, 2005; Sorensen, 2005). In addition to fast synchronous Ca^{2+}-triggered release, synapses exhibit slower asynchronous Ca^{2+}-triggered release that also depends on SNARE proteins but is independent of synaptotagmin 1 (Geppert et al., 1994; Deak et al., 2004).

Synaptotagmin 1 is a synaptic vesicle protein with two C_2 domains that both bind Ca^{2+} (Fernandez-Chacon et al., 2001; Fernandez et al., 2001). Biochemically, the C_2 domains of synaptotagmin 1 interact with negatively charged phospholipids and synaptic SNARE proteins (Perin et al., 1990; Bennett et al., 1992; Yoshida et al., 1992; Söllner et al., 1993; Davletov and Südhof, 1993; Li et al., 1995; Chapman et al., 1995; Banerjee et al., 1996; Schiavo et al., 1997; Fernandez et al., 2001; Rickman et al., 2004; Bowen et al., 2005). The interaction of synaptotagmin 1 with SNARE proteins has suggested a direct action of synaptotagmin 1 on SNARE complexes in triggering release. However, both Ca^{2+}-dependent and Ca^{2+}-independent interactions of synaptotagmin 1 with SNARE complexes have been described, and it is unclear which operate under physiological conditions (see references cited above).

In addition to synaptotagmin 1, SNARE complexes bind complexins (also called synaphins), small soluble proteins that insert as an antiparallel α helix into a groove of the four-helical bundle of the SNARE complex, but do not bind to individual SNARE proteins or to synaptotagmin 1 (McMahon et al., 1995; Ishizuka et al., 1995; Pabst et al., 2002; Bracher et al., 2002; Chen et al., 2002). In mice, deletion of complexins selectively impairs fast Ca^{2+}-triggered release, but not fusion in general (Reim et al., 2001). In particular, asynchronous slow Ca^{2+}-triggered release that is SNARE-complex dependent is not altered by deletion of complexins. Hence, although complexins

and synaptotagmin 1 do not appear to interact with each other, their deletions produce a similarly selective phenotype, suggesting that complexins and synaptotagmin 1 operate in the same pathway.

Not only deletion of complexins but also excess complexin inhibits exocytosis (Ono et al., 1998; Itakura et al., 1999; Archer et al., 2002; Giraudo et al., 2006; Schaub et al., 2006). For example, a peptide from the SNARE-binding sequence of complexin inhibits neurotransmitter release in microinjected squid synapses (Tokumaru et al., 2001). Although biophysical results rendered the specific molecular mechanism proposed in this study (SNARE-complex polymerization by complexin) unlikely (Bracher et al., 2002; Chen et al., 2002; Hu et al., 2002; Pabst et al., 2002), the overall conclusion mirrors that of other studies introducing an excess of complexin into cells, namely that complexins generally inhibit SNARE-dependent fusion. This conclusion, however, does not agree with the specific impairment of only fast Ca^{2+}-triggered release observed in complexin knockout (KO) mice (Reim et al., 2001).

In the present study, we demonstrate that complexins and synaptotagmin 1 act in the same pathway and compete with each other for SNARE-complex binding and that, similarly to the deletion of complexins, a local synaptic excess of complexin selectively blocks fast Ca^{2+}-triggered release, but not slow asynchronous release. These results, together with the earlier structural studies of the complexin-SNARE interaction (Chen et al., 2002), lead us to propose that complexins promote full assembly of SNARE complexes and simultaneously block completion of the fusion reaction, thus leading to a metastable state that is critical for synchronization of release during the short time window when Ca^{2+} concentrations are high. We hypothesize that primed metastable vesicles form the substrate for synaptotagmin 1, which triggers fast synchronous release upon Ca^{2+} influx by binding to SNARE complexes, displacing complexin, and coupling the SNARE complex to phospholipids. These studies suggest a mechanism not only for how synaptotagmin 1 acts in triggering release but also for how the metastable state of primed vesicles is maintained by complexins in preparation for synaptotagmin 1 action.

RESULTS

Binding of Purified Synaptotagmin 1 to SNARE Proteins

To examine the Ca^{2+}-dependent and Ca^{2+}-independent binding of synaptotagmin 1 to SNARE complexes, we immobilized GST-synaptobrevin on glutathione beads, reconstituted the SNARE complex on the beads in situ with recombinant SNARE motifs from SNAP-25 and syntaxin 1, and measured the Ca^{2+}-dependent binding of the recombinant C_2A/B domain fragment from synaptotagmin 1 (residues 140–421) in buffers containing 50–200 mM NaCl (Figures 1B and 1C). As a negative control, we used beads containing only GST-synaptobrevin (data not shown). We found that synaptotagmin 1 binding to SNARE complexes was activated at low and abolished at high ionic strengths. Binding was strictly Ca^{2+} dependent at intermediate, physiological ionic strengths but became at least partially Ca^{2+} independent at low ionic strengths (Figures 1B and 1C). We detected no binding of synaptotagmin 1 to GST-synaptobrevin alone (data not shown). The same experiment performed in the reverse orientation with immobilized GST-synaptotagmin yielded similar results, except that more Ca^{2+}-independent binding was observed (see Figure S1 in the Supplemental Data available with this article online). Titrations revealed that, in these binding reactions, synaptotagmin 1 and SNARE complexes formed stoichiometric complexes with a nanomolar affinity that was ~7-fold lower than that of the complexin-SNARE interaction (Figure S2). Viewed together, these data show that synaptotagmin 1 binds to assembled SNARE complexes in a precarious balance dictated by the ionic strength and the Ca^{2+} concentration and that, under nearly physiological conditions, Ca^{2+}-dependent binding is favored.

Synaptotagmin 1 and Complexin Compete for SNARE-Complex Binding

Since synaptotagmin 1 and complexin both bind to SNAREs, we asked whether they compete with each other for binding. We tested full-length wild-type complexin 1 (Cpx); full-length complexin 1 containing point mutations in the central SNARE-binding sequence (Cpx^M; mutations used for these and all experiments described below were R48A/R59A/K69A/Y70A); and N-terminally truncated complexin 1 that lacks the first 40 residues, which are not involved in SNARE-complex binding (Cpx^{Δ}). Full-length and truncated complexins bound tightly to SNARE complexes, whereas the mutant complexin did not (Figure S3). Titrations revealed that SNARE complexes were quantitatively displaced from synaptotagmin 1 by full-length complexin at submicromolar concentrations, but not by mutant complexin (Figures 1D–1E). Interestingly, N-terminally truncated complexin displaced only 50% of the SNARE complexes. The competition was observed independently of whether Ca^{2+} was present or absent, presumably because we carried out these experiments under saturating conditions that render SNARE binding to synaptotagmin 1 Ca^{2+} independent (Figure S1).

Characterization of the Ca^{2+}-Dependent Binding of Native Synaptotagmin 1 to Brain SNARE Complexes

To study the relation of complexin and synaptotagmin 1 binding to SNARE complexes with native proteins, we immunoprecipitated brain proteins solubilized in Triton X-100 (Figure 2). We immunoprecipitated SNARE complexes with syntaxin 1 or synaptobrevin 2 antibodies, measured the amounts of coimmunoprecipitated proteins by quantitative immunoblotting with ^{125}I-labeled secondary antibodies at different ionic strengths in the presence and absence of Ca^{2+}, and normalized the protein amounts for the immunoprecipitated protein to correct for

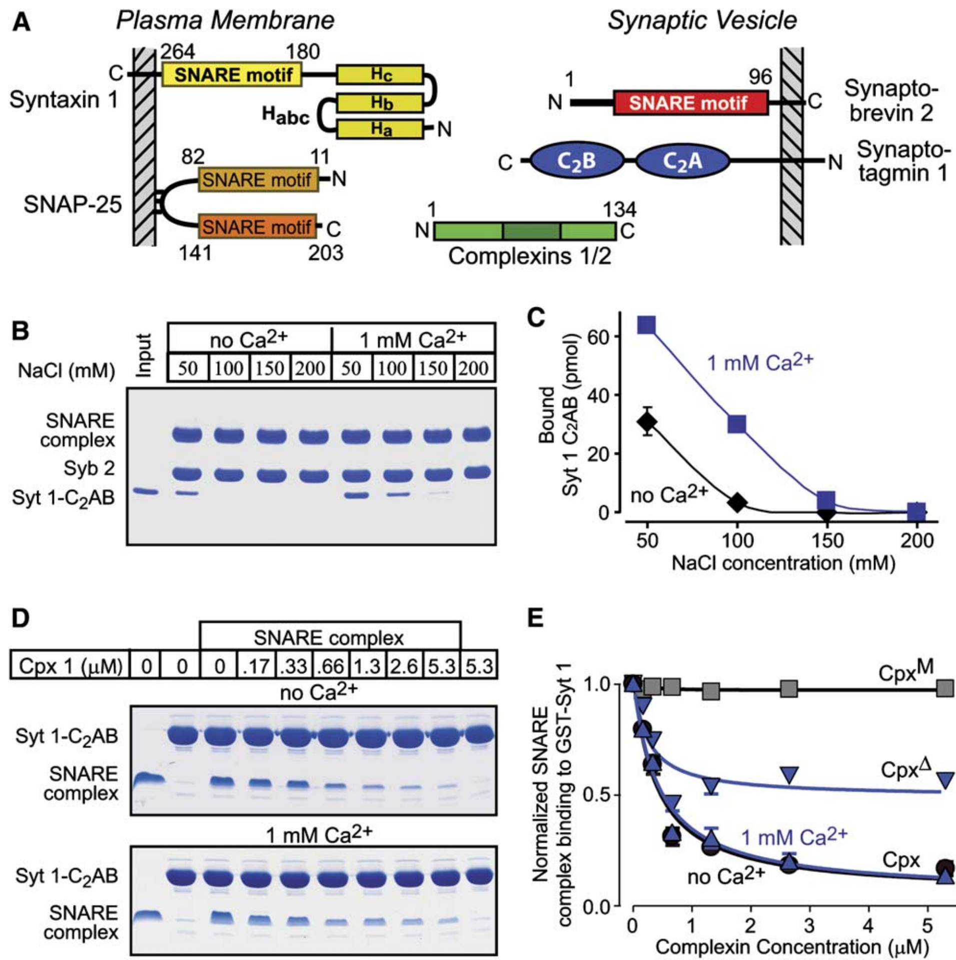

Figure 1. Competitive Binding of Synaptotagmin 1 and Complexin to SNARE Complexes

(A) Schematic diagrams of syntaxin 1 (yellow), SNAP-25 (orange), synaptobrevin 2/VAMP (red), synaptotagmin 1 (blue), and complexins (green).

(B and C) Effect of ionic strength on the binding of the double C_2 domain fragment from synaptotagmin 1 to immobilized SNARE complexes attached to the beads by excess GST-synaptobrevin. Binding was carried out with and without 1 mM free Ca^{2+}. Controls with GST-synaptobrevin alone exhibited no binding (data not shown). Data shown are means ± SEMs (n = 3).

(D and E) Displacement of SNARE complexes from synaptotagmin 1 by increasing concentrations of wild-type and mutant complexins (Cpx = full-length complexin 1; Cpx^{Δ} = N-terminally truncated complexin 1 lacking residues 1–40; Cpx^{M} = mutant full-length complexin 1 that does not bind to SNARE complexes; see Figure S3). (D) shows representative gels, while (E) depicts quantitations based on Coomassie-stained SDS gels (means ± SEMs; n = 3).

differences in yield. We found that, similar to the binding of recombinant proteins, binding of native synaptotagmin 1 to SNARE complexes was largely independent of Ca^{2+} at low ionic strength, predominantly Ca^{2+} dependent at intermediate (physiological) ionic strength, and inhibited at high ionic strength (Figure 2A).

Complexin Displaces Synaptotagmin 1 from Native Brain SNARE Complexes

To determine whether complexin and synaptotagmin 1 also compete with each other for binding to native SNARE complexes in solution, we performed the SNARE immunoprecipitations in the presence of increasing concentrations of exogenous complexins (Figure 2B). Without additions, the immunoprecipitates contained endogenous complexins. After addition of excess exogenous complexins, however, only exogenous complexins were present in the immunoprecipitated SNARE complex. (Note that the N-terminally truncated complexin 1 still binds effectively to SNARE complexes.) Rab3 and synaptophysin, analyzed as control proteins, were not coimmunoprecipitated.

We quantitated the displacement of synaptotagmin 1 from native SNARE complexes by complexin and also examined α-SNAP binding because α-SNAP is known to compete with complexin for binding to SNAREs (McMahon et al., 1995). Complexin binding was determined as a control to ensure that the exogenous complexin was incorporated into the precipitated complex. Nanomolar complexin concentrations displaced >80% of synaptotagmin 1 from the immunoprecipitated SNARE complexes.

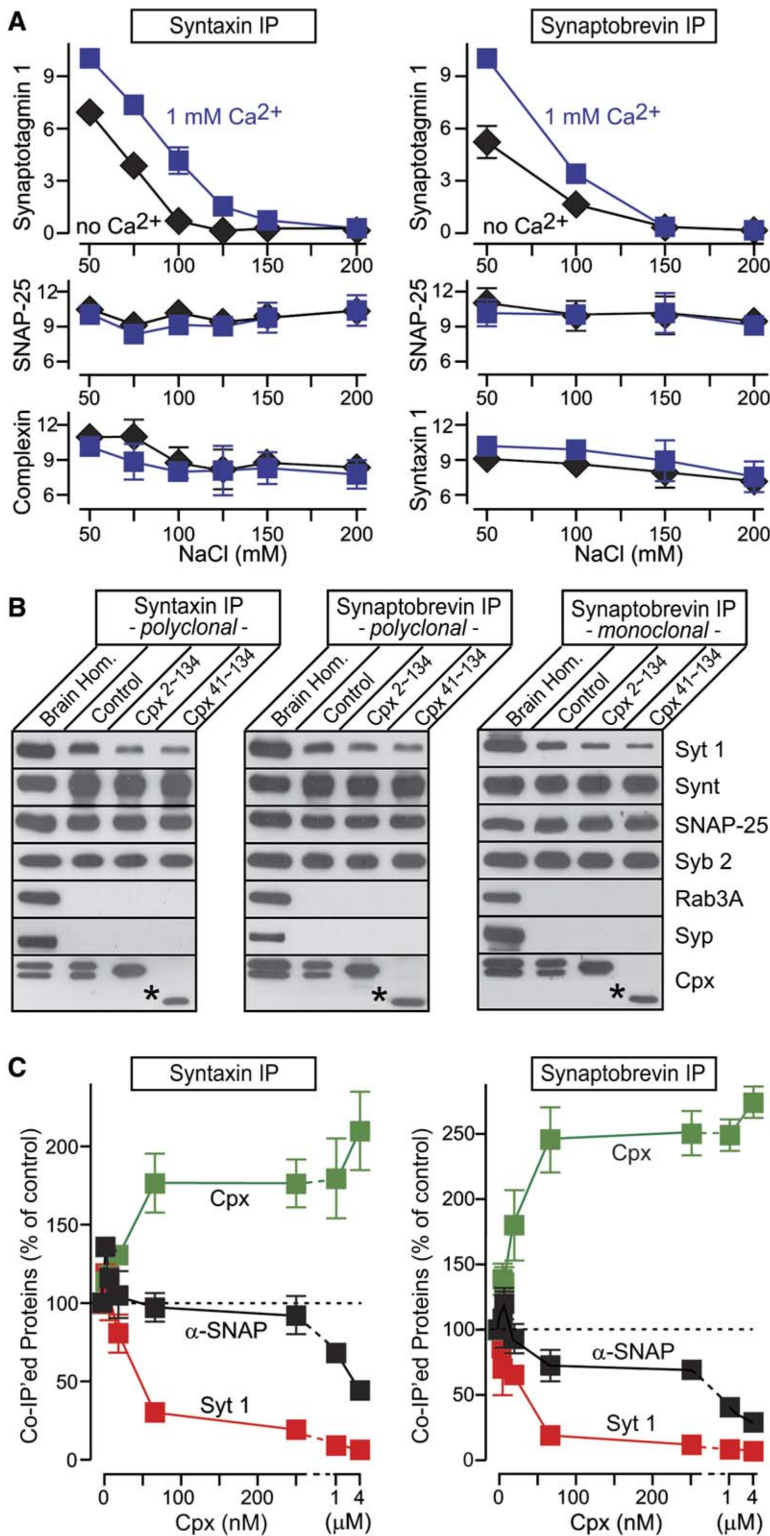

Figure 2. Immunoprecipitation Analysis of Synaptotagmin 1 and Complexin Binding to Native SNARE Complexes

(A) Effect of ionic strength and Ca^{2+} on synaptotagmin 1 binding to native brain SNARE complexes. SNARE complexes were immunoprecipitated at the indicated NaCl concentrations with antibodies to syntaxin 1 or synaptobrevin 2 with or without 1 mM free Ca^{2+}. Immunoprecipitated proteins were quantified by immunoblotting using ^{125}I-labeled secondary antibodies and phosphorimager detection and were normalized for the amount of immunoprecipitated syntaxin 1 (n = 4) and synaptobrevin 2 (n = 3). Data shown are means ± SDs.

(B) Representative immunoprecipitations from brain homogenates with polyclonal syntaxin 1 antibodies (left panel) and polyclonal and monoclonal synaptobrevin 2 antibodies (central and right panels) either without addition or after addition of full-length (Cpx^{2-134}) or N-terminally truncated complexin 1 (Cpx^{41-134}; both 13.2 μM). Immunoprecipitates were immunoblotted with antibodies to synaptotagmin 1 (Syt 1), syntaxin 1 (Synt), SNAP-25, synaptobrevin 2 (Syb 2), Rab3A, synaptophysin (Syp), and complexin (Cpx; asterisk = position of the truncated complexin that is coimmunoprecipitated).

(C) Amounts of complexins, α-SNAP, and synaptotagmin 1 coimmunoprecipitated with SNARE complexes with antibodies to syntaxin 1 (left) or to synaptobrevin 2 (right) in 1 mM Ca^{2+} in the presence of increasing concentrations of exogenous complexin 1. The relative amounts of complexins, α-SNAP, and synaptotagmin 1 in the immunoprecipitates were measured by quantitative immunoblotting (means ± SDs; n = 4 independent experiments). Control experiments showed that addition of up to 5 μM recombinant complexin 1 did not significantly change the amount of syntaxin 1 or SNAP-25 in the immunoprecipitates (data not shown).

In contrast, complexin was less effective in displacing α-SNAP from SNARE complexes (Figure 2C), consistent with the idea that α-SNAP has a higher affinity for SNARE complexes than complexin and synaptotagmin 1. In these experiments, the complexin concentrations required for displacement of synaptotagmin 1 from SNARE complexes were lower than in the recombinant protein experiments (Figure 1E), possibly because the synaptotagmin 1

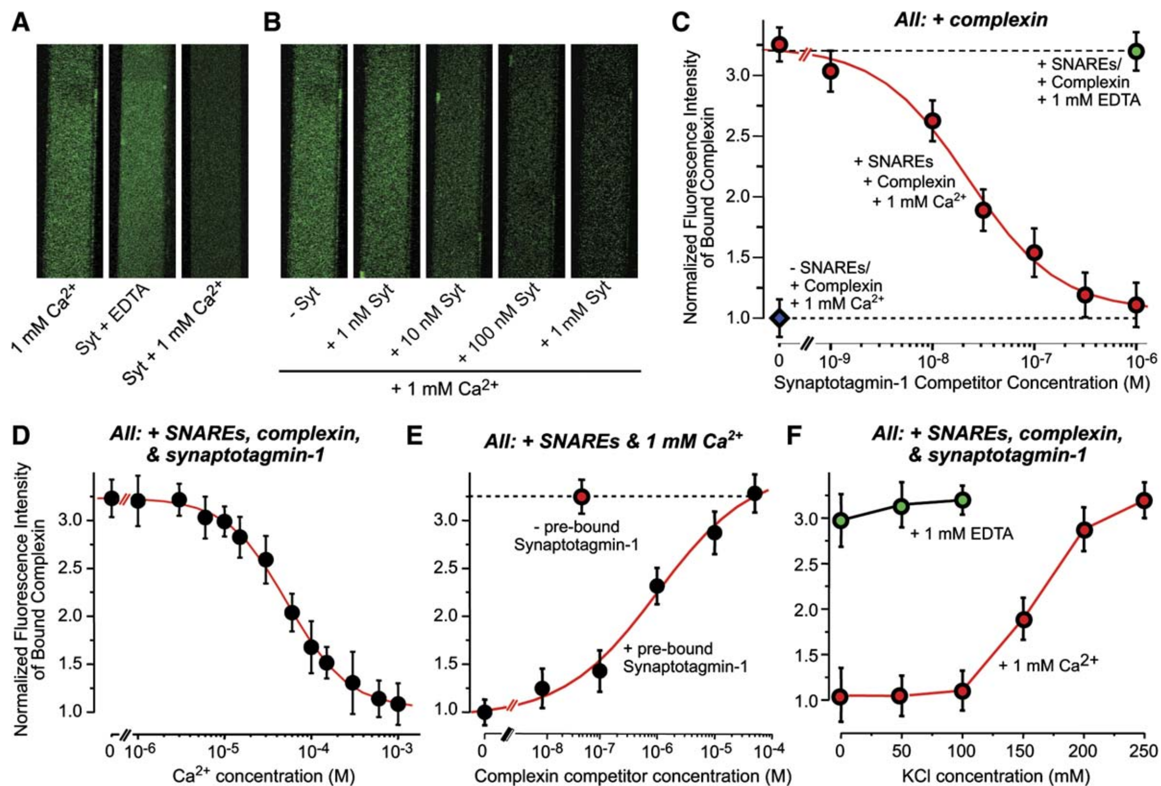

Figure 3. Synaptotagmin 1 Displaces Complexin from Membrane-Attached SNARE Complexes in a Ca^{2+}-Dependent Manner

(A) Confocal micrographs of supported bilayers containing reconstituted SNARE complexes loaded with 50 nM fluorescent complexin (residues 26–83; labeled with BODIPY-FL). Bilayers were deposited in microfluidic channels (0.2 mm width) and washed with buffer containing 100 mM KCl and the indicated additions (Syt = 1 μM synaptotagmin 1 cytoplasmic region).

(B) An analogous experiment in which SNARE complexes in the bilayer were loaded with fluorescent complexin and then washed with Ca^{2+}-containing buffer and increasing concentrations of synaptotagmin 1 as indicated. Each wash with synaptotagmin 1 was followed by a wash with buffer alone.

(C) Displacement of prebound fluorescent complexin from SNARE complexes by synaptotagmin 1. Experiments were performed as in (B); average fluorescence intensities measured under each condition were normalized to the control, in which fluorescently labeled complexin was added to a supported bilayer lacking SNARE complexes (blue diamond). Without Ca^{2+}, even the maximal synaptotagmin 1 concentration cannot displace fluorescent complexin from the SNARE complexes (green circle). Data were fitted to a dose-response curve (EC_{50} = 23 ± 5 nM synaptotagmin 1; Hill coefficient = −0.96 ± 0.02 [n = 3 independent experiments]). Data shown in (C)–(F) are means ± SDs.

(D) Ca^{2+} titration of the displacement of fluorescent complexin from SNARE complexes by 1 μM synaptotagmin 1. Experiments were performed as in (C) with increasing concentrations of free Ca^{2+} (EC_{50} = 53 ± 14 μM Ca^{2+}; Hill coefficient = −1.24 ± 0.37).

(E) Displacement of prebound synaptotagmin 1 from SNARE complexes by fluorescent complexin. The displacement of synaptotagmin 1 bound to the reconstituted SNARE complexes was measured in 1 mM Ca^{2+} as the binding of increasing concentrations of fluorescent complexin (EC_{50} = 1.00 ± 0.65 μM; Hill coefficient = −0.53 ± 0.18). As a control, the red circle indicates the amount of 50 nM fluorescent complexin bound in the absence of prebound synaptotagmin 1.

(F) Effect of ionic strength on the displacement of bound complexin from SNARE complexes by synaptotagmin 1. SNARE complexes were loaded with fluorescent complexin, and the amount of complexin remaining after washing with 1 μM synaptotagmin 1 at different KCl concentrations was quantified. Experiments were performed in the presence of 1 mM Ca^{2+} (red circles) or 1 mM EDTA (green circles).

concentration is lower in brain homogenates or because complexin binding to SNARE complexes is facilitated when SNARE proteins contain transmembrane regions (Hu et al., 2002; however, see Bowen et al., 2005).

Ca^{2+}-Dependent Displacement of Complexin by Synaptotagmin 1 in a Membrane-Attached Environment

SNARE proteins and synaptotagmin 1 are normally embedded in membranes, whereas all experiments up until now were performed in a nonmembranous environment. To examine the role of Ca^{2+} in the binding of synaptotagmin 1 to SNARE complexes in a physiological membranous environment, and to test whether synaptotagmin 1 can displace complexin from SNARE complexes (and not only whether complexin can displace synaptotagmin 1), we reconstituted membrane-bound SNARE complexes into a supported bilayer deposited within a microfluidic channel. We then bound fluorescently labeled complexin to the complexes and measured the ability of recombinant synaptotagmin 1 to displace complexin from the SNARE complexes as a function of Ca^{2+} (Figure 3A).

Synaptotagmin 1 quantitatively displaced complexin from SNARE complexes in the presence, but not the absence, of Ca^{2+}. In displacing complexin, synaptotagmin 1 exhibited an EC_{50} of 23 ± 5 nM (Figures 3A–3C) and a micromolar apparent Ca^{2+} affinity (EC_{50} = 53 ± 14 μM; Figure 3D). All of these experiments were performed in a nearly physiological salt solution (100 mM KCl and 25 mM HEPES-NaOH), suggesting that, in a membranous environment, synaptotagmin 1 powerfully dislodges complexin from SNARE complexes. To probe whether complexin in a membranous environment can in turn displace synaptotagmin 1, we measured the binding of fluorescently labeled complexin to membrane-attached SNARE complexes containing prebound synaptotagmin 1 (Figure 3E). Without prebound synaptotagmin 1, 50 nM complexin quantitatively bound to the SNARE complexes. With prebound synaptotagmin 1, by contrast, micromolar complexin concentrations were required for binding, with an apparent affinity ~40-fold lower than that of the displacement of complexin by synaptotagmin 1 (EC_{50} = 1.0 ± 0.2 μM). Similar to the GST pull-downs and immunoprecipitation experiments, high ionic strength inhibited the displacement of complexin from SNARE complexes by 1 μM synaptotagmin 1 (Figure 3F). As opposed to these experiments, however, low ionic strength (0 and 50 mM KCl) did not enable synaptotagmin 1 to displace complexin from SNARE complexes in a Ca^{2+}-independent manner. This suggests that, in a membranous environment, lowering the ionic strength does not activate Ca^{2+}-independent binding of synaptotagmin 1 to SNARE complexes. Overall, these data show that, under close to physiological conditions in a membranous environment, synaptotagmin 1 binds to SNARE complexes in a strictly Ca^{2+}-dependent manner and effectively displaces complexin from SNARE complexes.

Synaptically Targeted Complexin/Synaptobrevin Fusion Protein Inhibits Spontaneous Synaptic Activity

A possible interpretation of the biochemical data is that complexins serve as fusion clamps that are "unclamped" when Ca^{2+} binding to synaptotagmin 1 triggers the displacement of complexin from SNARE complexes. A similar hypothesis was advanced in experiments in which transfected cells that displayed surface-exposed SNARE proteins or liposomes with reconstituted SNARE proteins were fused in vitro; in both cases, addition of excess complexin inhibited fusion, consistent with a clamp function for complexin (Giraudo et al., 2006; Schaub et al., 2006). However, the clamp hypothesis is at odds with the complexin KO phenotype, which demonstrates that complexins are activators, not inhibitors, of fusion (Reim et al., 2001). It is possible that the KO analyses are misleading because of compensatory effects or that the in vitro fusion experiments are misleading because the assays using surface-exposed SNAREs in transfected cells or liposomes do not measure a normal synaptic membrane-fusion reaction. Thus, to directly test the effect of excess complexin on SNARE-mediated synaptic membrane fusion, we constructed a complexin/synaptobrevin fusion protein that increases the local concentration of complexin at the synapse. This fusion protein is composed of an N-terminal complexin, a central synaptobrevin, and a C-terminal Venus-YFP (Figure 4A). Complexin is separated from synaptobrevin by a flexible linker to allow folding back of complexin onto the SNARE complex into which the synaptobrevin has been incorporated, thereby increasing the local concentration of complexin at the site of synaptic SNARE-complex formation. Experiments with recombinant proteins confirmed that the complexin/synaptobrevin fusion protein blocks synaptotagmin 1 binding to SNARE complexes but has no effect on α-SNAP binding (Figure S4). In contrast, a fusion protein with mutant complexin that does not bind to SNARE complexes fully allowed synaptotagmin 1 binding.

We expressed the complexin/synaptobrevin proteins in dissociated cortical neurons at day 5 in vitro using lentiviral delivery. Immunoblots revealed that these proteins were expressed at levels similar to those of endogenous synaptobrevin (Figure 4B). Imaging documented that the complexin/synaptobrevin proteins were highly concentrated in presynaptic terminals, where they colocalized with FM5-95, an activity-dependent marker of synaptic vesicles (Figure 4C and Figure S5). We analyzed the effect of the complexin/synaptobrevin fusion protein on synaptic transmission using whole-cell voltage-clamp recordings on day 14–16 in culture, when neurons display robust postsynaptic responses to extracellular stimulation (Maximov and Südhof, 2005). Recordings of spontaneous release events revealed that the wild-type but not the mutant fusion protein reduced the rate of spontaneous mini events ~3-fold (Figures 4D and 4E) but had no effect on the size of spontaneous mini events (Figure 4F), consistent with a presynaptic role in release.

The Complexin/Synaptobrevin Fusion Protein Inhibits Fast Ca^{2+}-Triggered Neurotransmitter Release

To determine which modes of release are perturbed by the local increase in the concentration of complexin in synapses, we measured inhibitory postsynaptic currents (IPSCs) in the presence of glutamate-receptor blockers (50 μM APV and 20 μM CNQX). We compared IPSCs obtained in noninfected wild-type neurons, in neurons from synaptotagmin 1 and synaptobrevin 2 KO mice, and in wild-type neurons expressing complexin/synaptobrevin proteins. Initially, we analyzed responses to single action potentials (Figure 5A). Expression of the full-length or N-terminally truncated complexin/synaptobrevin fusion proteins led to an 80% depression of the amplitude and the charge transfer of the IPSCs (Figure 5B and 5C). The control complexin/synaptobrevin fusion protein with a mutation that inactivates SNARE-complex binding by complexin had no significant effect on the size of the IPSCs, demonstrating that the SNARE-binding activity of complexin is responsible for the inhibitory effect. The inhibitory

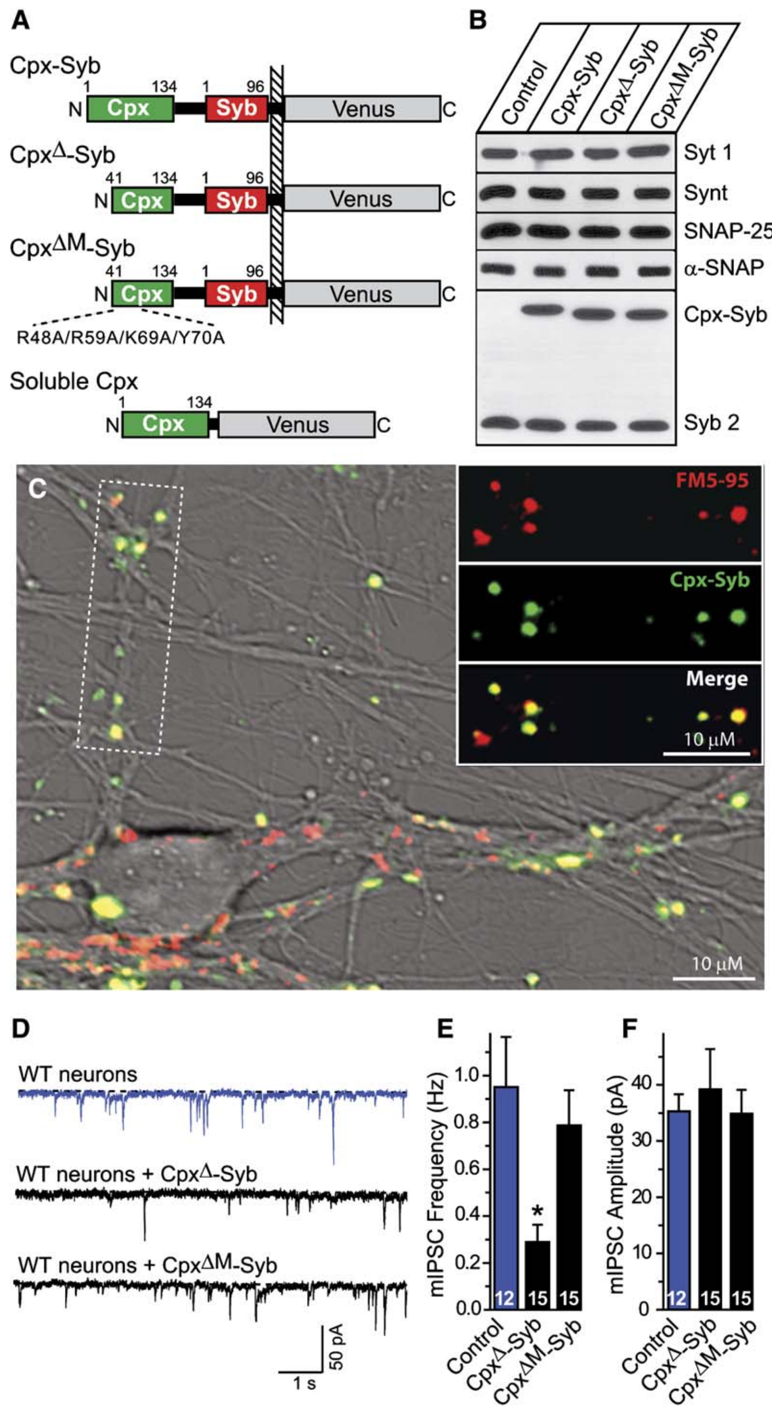

Figure 4. Expression and Localization of Complexin/Synaptobrevin Fusion Protein in Cultured Cortical Neurons: Effect on Spontaneous Release Events

(A) Schematic diagram of the structure of complexin (Cpx) and complexin/synaptobrevin 2 fusion proteins. Expressed proteins contain full-length complexin 1 (Cpx and Cpx-Syb), N-terminally truncated wild-type complexin 1 (Cpx^{Δ}-Syb; residues 41–134), and N-terminally truncated mutant complexin 1 that lacks SNARE binding ($Cpx^{\Delta M}$-Syb with the R48A/R59A/K69A/Y70A mutation; Figure S3). Complexin is fused to full-length synaptobrevin with a 49 residue linker, and fluorescent Venus protein is fused C-terminally to complexin or Cpx-Syb.

(B) Immunoblot analysis of various synaptic proteins (as indicated on the right) and Cpx-Syb fusion proteins in cortical neurons infected with Cpx-Syb lentiviruses.

(C) Localization of the Cpx^{Δ}-Syb fusion protein (visualized via its Venus fluorescence, green) in neurons whose synapses were loaded with FM5-95 (red). Fluorescent images were overlaid with bright-field DIC to visualize neuronal cell bodies and processes. Insets illustrate colocalization of Cpx^{Δ}-Syb with FM5-95-positive synaptic puncta in the region marked by a white box on the left (see also Figure S5).

(D) Representative traces of miniature inhibitory postsynaptic currents (mIPSCs) monitored in 1 μM tetrodotoxin, 20 μM CNQX, and 50 μM AP5 from noninfected wild-type neurons or wild-type neurons expressing Cpx^{Δ}-Syb or $Cpx^{\Delta M}$-Syb fusion proteins. Scale bars apply to all traces.

(E and F) Average frequency (E) and amplitude (F) of mIPSCs monitored in noninfected neurons or neurons expressing Cpx^{Δ}-Syb or $Cpx^{\Delta M}$-Syb (means ± SEMs; numbers of neurons analyzed [n] in three independent cultures are indicated in the bars).

effect of the wild-type complexin/synaptobrevin protein was indistinguishable from that of the synaptotagmin 1 deletion, but less than that of the synaptobrevin 2 deletion, presumably because the asynchronous release remains in the synaptotagmin 1 KO but is depressed together with the synchronous release in synaptobrevin 2 KO mice (Geppert et al., 1994; Deak et al., 2004).

Constitutive Complexin Binding to SNARE Complexes Does Not Block Asynchronous Release

Does transient expression of the complexin/synaptobrevin fusion protein fully impair exocytosis, similar to the deletion of synaptobrevin 2 as suggested by the global overexpression studies, or does it specifically block exocytosis triggered by Ca^{2+} binding to synaptotagmin 1 as

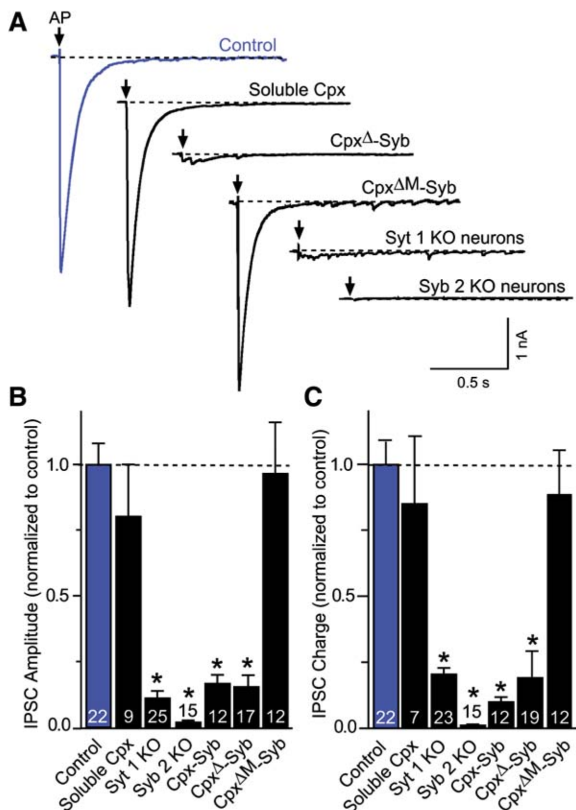

Figure 5. Expression of a Complexin/Synaptobrevin Fusion Protein in Wild-Type Neurons Impairs Synaptic Responses Triggered by Single Action Potentials

(A) Representative IPSCs recorded from noninfected wild-type neurons; synaptotagmin 1- and synaptobrevin 2-deficient neurons; and wild-type neurons infected with lentiviruses encoding soluble complexin, Cpx^{Δ}-Syb, or $Cpx^{\Delta M}$-Syb. IPSCs were triggered by isolated APs at 0.1 Hz (induced by local extracellular stimulation of adjacent neurons) and were recorded in whole-cell voltage-clamp mode in CNQX (20 μM) and AP5 (50 μM). Scale bars apply to all traces.

(B and C) Average amplitudes (B) and total charge transfers integrated over 1.5 s (C) of IPSCs recorded from wild-type neurons, synaptotagmin 1- and synaptobrevin 2-deficient neurons, and wild-type neurons expressing the indicated complexin/synaptobrevin fusion proteins. Numbers of neurons analyzed in each group are indicated in the bars, data are from at least two independent cultures, and values are normalized for the control analyzed in the same experiment. Data shown are means ± SEMs.

suggested from the analysis of the complexin knockout? To distinguish between these two possibilities, we examined the effects of the complexin/synaptobrevin fusion proteins on release triggered by trains of action potentials (100 APs at 10 Hz). In synaptobrevin 2 KO neurons, synaptic responses remain suppressed during high-frequency stimulus trains (Deak et al., 2004), with a total amount of release that was <5% of the release of wild-type controls over the entire stimulus train (Figure 6A). In synaptotagmin 1-deficient neurons, in contrast, synaptic responses are only initially abnormal during a high-frequency stimulus train but quickly reach wild-type levels, and the total amount of release over the entire train is not significantly different from wild-type neurons (Maximov and Südhof, 2005).

Analysis of the release produced during high-frequency trains in neurons expressing complexin/synaptobrevin proteins revealed a pattern indistinguishable from that observed in synaptotagmin 1 KO neurons: The initial fast synchronous responses were absent, but subsequent asynchronous release that becomes dominant after three to five action potentials was normal (Figure 6B). Quantitation of the total synaptic charge transfer during the stimulus train confirmed that in contrast to the synaptobrevin 2 KO neurons, neurons lacking synaptotagmin 1 or expressing the complexin/synaptobrevin fusion protein exhibited nearly normal asynchronous release (Figure 6C). This release is indeed asynchronous because it is blocked by EGTA-AM (Figure 6B), as previously demonstrated for asynchronous release in wild-type neurons (Lu and Trussell, 2000) and synaptotagmin 1 KO neurons (Maximov and Südhof, 2005). Moreover, plots of the time course of cumulative charge transfer during the stimulus train demonstrate that the initial four to five action potentials induced little synaptic charge transfer in either synaptotagmin 1 KO neurons or wild-type neurons expressing the wild-type complexin/synaptobrevin protein. Later action potentials, however, induce normal synaptic charge transfers in these neurons (Figure 6D). Finally, initial responses were desynchronized in synaptotagmin 1 KO neurons and wild-type neurons expressing the wild-type complexin/synaptobrevin protein, but not in wild-type neurons expressing the mutant complexin/synaptobrevin protein (Figure 6E). This is evident from the fact that the first ten responses during the stimulus train were highly synchronized in wild-type control neurons and neurons expressing mutant complexin/synaptobrevin fusion protein, as shown by the uniformly short rise times (although the mutant complexin/synaptobrevin fusion protein did have a moderate effect on rise times). In contrast, neurons expressing wild-type complexin/synaptobrevin fusion protein exhibited massive desynchronization as reflected in a scattering of the rise times (Figure 6E).

DISCUSSION

Neurotransmitter release is an exquisitely regulated process that exhibits a fast synchronous and a slower asynchronous Ca^{2+}-triggered component (Goda and Stevens, 1994; Südhof, 2004). The basic machinery for fast synchronous release is composed of SNARE proteins, which mediate fusion; synaptotagmins, which serve as Ca^{2+} sensors; and complexins, which bind to SNARE complexes (Geppert et al., 1994; Fernandez-Chacon et al., 2001; Reim et al., 2001). Whereas SNAREs are also required for slow asynchronous release, synaptotagmins and complexins are not. These data suggest that, after synaptic vesicles are primed for release by SNARE-complex assembly, two alternative pathways of release

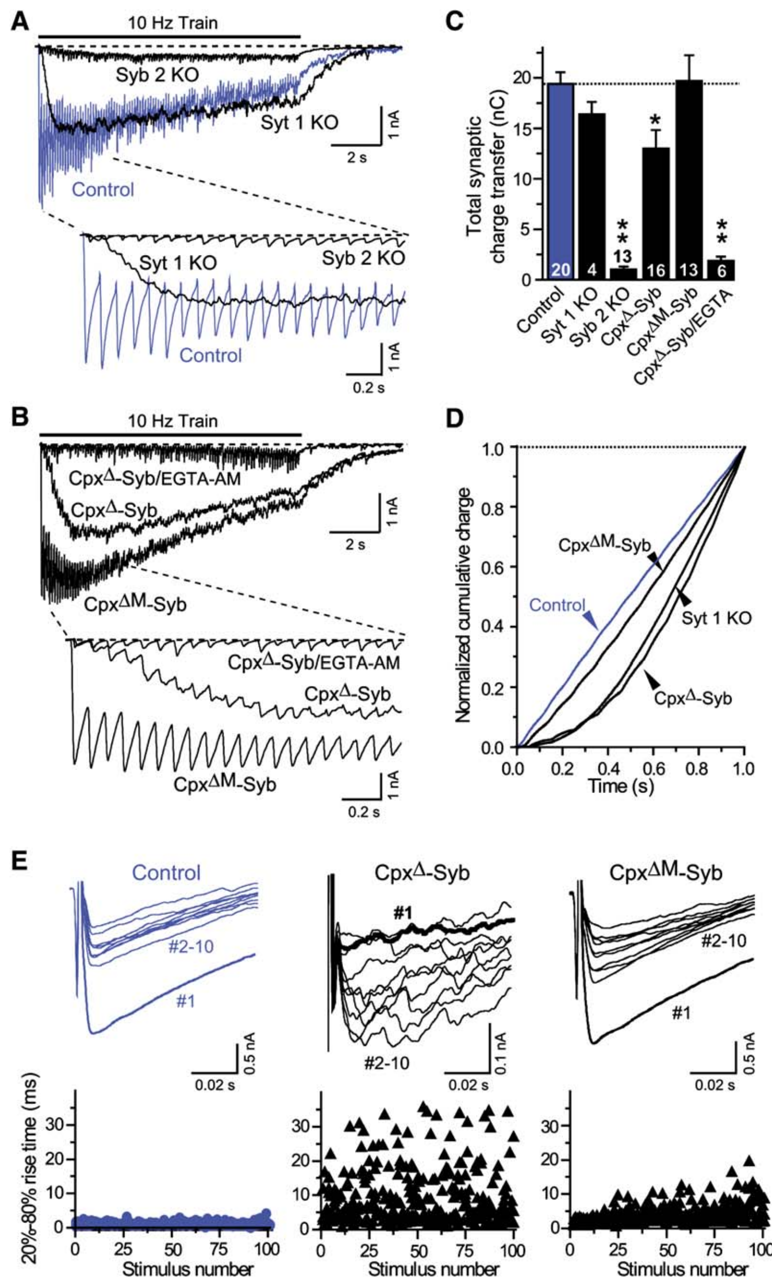

Figure 6. Expression of a Complexin/Synaptobrevin Fusion Protein Causes Loss of Fast Ca^{2+}-Triggered Release but Not of Asynchronous Ca^{2+}-Triggered Release

(A) Representative IPSCs during a 10 Hz stimulus train for 10 s from wild-type neurons (Control), synaptotagmin 1-deficient neurons (Syt 1 KO), or synaptobrevin 2-deficient neurons (Syb 2 KO).

(B) IPSCs monitored in the same conditions from nontreated wild-type neurons expressing Cpx^{Δ}-Syb and $Cpx^{\Delta M}$-Syb or neurons expressing Cpx^{Δ}-Syb and treated for 5 min with 0.1 mM EGTA-AM. In (A) and (B), expanded lower traces illustrate the initial onsets of high-frequency responses (filtered at 50 Hz to remove the stimulus artifacts). Scale bars apply to all traces.

(C) Total synaptic charge transfer during a 10 Hz stimulus train for 10 s monitored in noninfected wild-type neurons, synaptotagmin 1 KO and synaptobrevin 2 KO neurons, or neurons infected with Cpx-Syb lentiviruses (analyzed neuron numbers are indicated in the bars; $*p < 0.01$; $**p < 0.001$). Data shown are means ± SEMs.

(D) Average plots of the normalized cumulative charge transferred during the first 1 s of 10 Hz stimulation shown in (A) and (B).

(E) Top: aligned segments of the initial 10 IPSCs during a 10 Hz stimulus train to illustrate that the synchronous responses become irregular in neurons expressing Cpx^{Δ}-Syb. Bottom: plots of the 20%–80% rise times of individual IPSCs triggered during a 10 Hz stimulus train in control wild-type neurons and in neurons expressing Cpx^{Δ}-Syb or $Cpx^{\Delta M}$-Syb. For each group, data are from three different neurons.

operate: a fast synchronous-release pathway that requires synaptotagmins and complexins and dominates whenever there is no accumulation of residual Ca^{2+} (e.g., during low-frequency stimulation or in the presence of high Ca^{2+} buffer concentrations), and an asynchronous-release pathway that dominates during repetitive stimulation in the presence of increased bulk Ca^{2+}. Although previous observations defined complexins as essential coactivators for synaptotagmin 1 in Ca^{2+}-triggered fast synchronous release, no biochemical connection between complexins and synaptotagmin 1 was known.

Several studies, including recent membrane-fusion experiments using cells that display surface-exposed SNARE proteins (Giraudo et al., 2006) or liposomes that contain reconstituted SNARE proteins (Schaub et al., 2006), have proposed that complexins serve as fusion clamps and that Ca^{2+} binding to synaptotagmin 1 relieves this clamp by an unknown biochemical mechanism. This proposal, however, is inconsistent with the phenotype of the complexin KO mice, which suggests that complexins are activators of fast synchronous release and do not function as general clamps for SNARE-dependent fusion (Reim et al., 2001). Moreover, the proposal that complexins are general clamps for SNARE-dependent fusion is incompatible with the present biochemical and physiological data. Our biochemical results reveal a selective

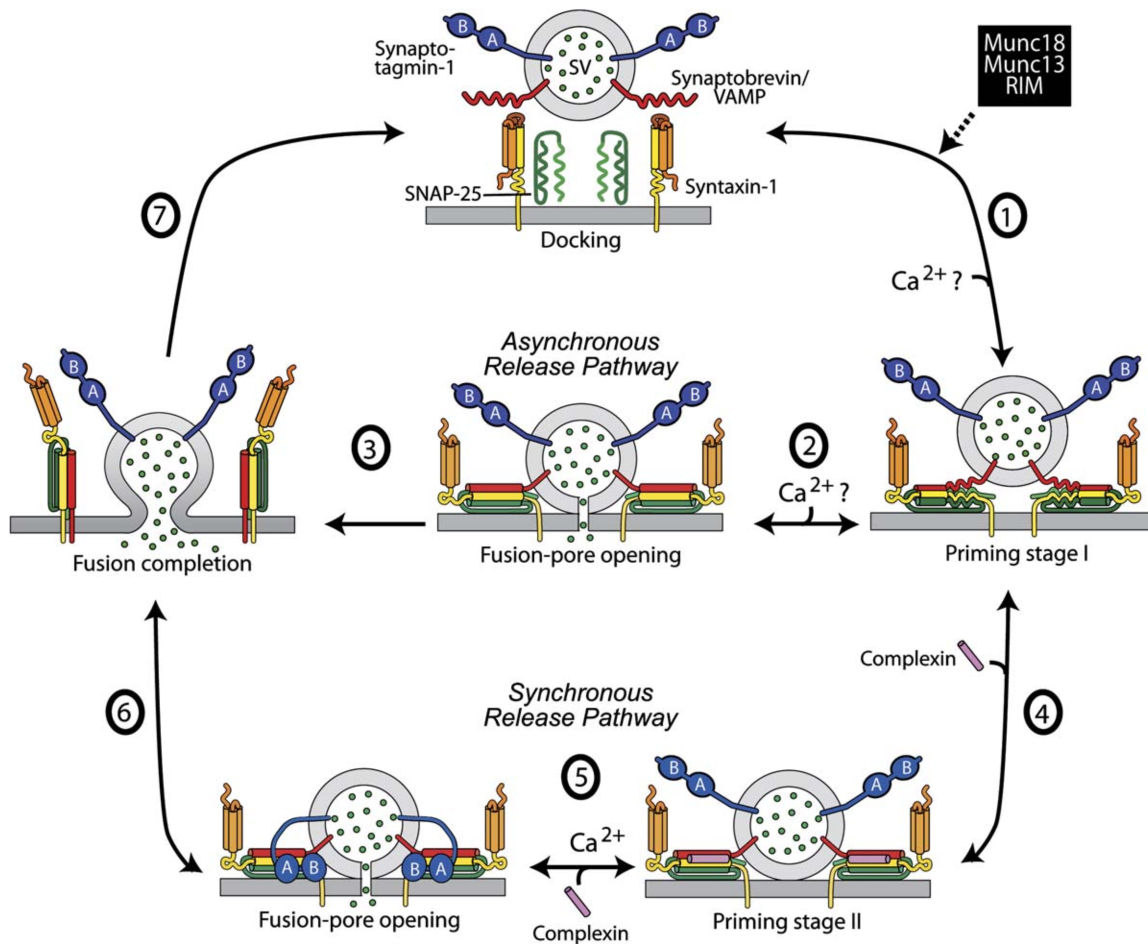

Figure 7. Model for Complexin and Synaptotagmin 1 Function in Ca^{2+}-Triggered Release

Docked vesicles containing unassembled SNARE complexes (top) are primed for release by partial SNARE-complex assembly catalyzed by Munc18, Munc13, and RIM (step 1). The resulting primed vesicles form the substrate for two release pathways: asynchronous release, in which full assembly of the SNARE complexes leads to fusion-pore opening followed by complete fusion (steps 2 and 3), and synchronous release, in which "superpriming" by binding of complexins to assembled SNARE complexes (step 4) activates and freezes SNARE complexes in a metastable state (referred to as priming stage 2). This stage is then substrate for fast Ca^{2+} triggering of release when Ca^{2+} binding to synaptotagmin 1 induces its binding to phospholipids and to SNARE complexes, with the latter reaction displacing complexin and resulting in fusion-pore opening (step 5). Again, opened fusion pores can then dilate to complete fusion (step 6), although both steps 2 and 5 are potentially reversible—i.e., lack of dilation of the fusion pore could lead to "kiss-and-run" exocytosis in these pathways. Note that steps 1 and 4 are also probably reversible, with a much faster forward than backward speed. It is likely that step 1 is Ca^{2+} dependent, but it is unclear whether or not step 2 is Ca^{2+} dependent since it is possible that asynchronous release is Ca^{2+} dependent solely because Ca^{2+} accelerates step 1, and step 2 has a finite probability. Thus, Ca^{2+} triggering of asynchronous release could operate at the priming and/or the actual fusion step.

competition of complexins with synaptotagmin 1 for SNARE-complex binding, consistent with a functional coupling between complexins and synaptotagmin 1 (which in turn is linked only to synchronous release, not to general SNARE-dependent fusion). Our physiological results demonstrate that a local excess of complexin inhibits fast synchronous release, presumably by outcompeting synaptotagmin 1, but has little effect on SNARE-dependent asynchronous release.

We suggest an alternative interpretation of these data (see model in Figure 7). Taking into consideration that earlier structural studies have suggested that complexins stabilize the fully assembled SNARE complex (Chen et al., 2002), we propose that complexins, by binding to SNARE complexes that have been partially assembled during priming, force completion of SNARE-complex assembly and thereby transform vesicles into a "superprimed" metastable state. According to this hypothesis, complexins activate vesicles by converting SNARE complexes from a loose to a tightly assembled state that serves as essential substrate for subsequent synaptotagmin 1 function. Complexin-activated vesicles are then triggered to undergo fusion when Ca^{2+} binding to synaptotagmin 1 stimulates simultaneous binding of synaptotagmin 1 to

SNARE complexes and phospholipids, thereby displacing complexin. This hypothesis suggests that complexin is required for synaptotagmin 1 function, as revealed in the complexin KO mice, because the metastable vesicle state that is the substrate for synaptotagmin 1 action is dependent on complexin. The evidence for this hypothesis is as follows:

1. We show that synaptotagmin 1 binding to SNARE complexes is controlled by a precarious balance of ionic strength and Ca^{2+}, providing an explanation for the contradictions between previous studies about the Ca^{2+} dependence of synaptotagmin 1 binding to SNARE complexes. At physiological ionic strength, synaptotagmin 1 binds to SNARE complexes in a Ca^{2+}-regulated manner, although we cannot rule out that the high local concentrations of synaptotagmin 1 at the synapse render at least some binding Ca^{2+} independent (see Figure 1, Figure 2, Figure 3, and Figures S1 and S2). At low ionic strength, synaptotagmin 1 binding to SNARE complexes is largely independent of Ca^{2+} in solution (Figure 1 and Figure 2) but remains Ca^{2+} dependent in a membranous environment (Figure 3). At high ionic strength, no syntaptotagmin-1 binding to SNARE complexes is observed under any condition. The strong Ca^{2+} dependence of synaptotagmin 1 binding to SNARE complexes implies that complexin binding to SNARE complexes dominates prior to the action-potential-driven local increase in Ca^{2+} at the terminal that leads to release (see below).
2. Complexin and synaptotagmin 1 compete for binding to SNARE complexes. In the context of membrane-bound SNARE complexes, synaptotagmin 1 is ~40-fold more potent in displacing complexin from SNARE complexes than complexin is in displacing synaptotagmin 1 (Figure 3). The binding competition between complexin and synaptotagmin 1 shown here contradicts our previous finding that complexin and synaptotagmin 1 can be coimmunoprecipitated with each other (McMahon et al., 1995). However, the previous coimmunoprecipitations were analyzed by sensitive but nonquantitative immunoblotting methods, which would make trace amounts of bound protein (which could, for example, be present because of SNARE-complex oligomerization) appear to represent significant binding. In contrast, for the present studies, we employed assays that measure stoichiometric binding, making the results more accurate.
3. At physiological ionic strength, displacement of complexin from SNARE complexes by synaptotagmin 1 is absolutely dependent on Ca^{2+}, presumably because synaptotagmin 1 binding to SNARE complexes under these conditions requires Ca^{2+} (Figure 3). Moreover, the apparent Ca^{2+} affinity of the Ca^{2+}-dependent displacement of complexin from SNARE complexes is in the physiological range of neurotransmitter release (~50 μM; Figure 3).
4. Increasing the local concentration of complexin by fusing it to synaptobrevin selectively suppresses fast neurotransmitter release, creating a phenocopy of the synaptotagmin 1 KO (Figure 5 and Figure 6). This result is predicted by our model (Figure 7), which suggests that complexin acts as an activator of SNARE complexes that shares an overlapping SNARE-complex binding site with synaptotagmin 1. Indeed, increases in the local synaptic complexin concentration selectively suppress synaptotagmin 1 function, but—consistent with the model (Figure 7)—have little effect on SNARE-dependent asynchronous release (Figure 5 and Figure 6). This selective phenotype depends on the ability of complexin to bind to SNARE complexes.
5. Considerable independent evidence in the literature supports the presence of superprimed vesicles. For example, different populations of primed vesicles were defined physiologically in the calyx of Held synapse (Trommershauser et al., 2003) and in hippocampal synapses (Schlüter et al., 2004) and were hypothesized to involve different states of SNARE-complex assembly in chromaffin cells (Xu et al., 1999).

The crystal structure of the complex between complexin and SNARE complexes (Chen et al., 2002) suggests a mechanism by which complexins may activate vesicles into a superprimed metastable state. Complexins bind to and stabilize the C-terminal, membrane-proximal half of SNARE complexes, indicating that, by forcing completion of SNARE-complex assembly, complexins may induce a strain on the participating membranes in which the respective SNARE proteins reside (i.e., in the case of the synapse, synaptobrevin on synaptic vesicles and syntaxin on the plasma membrane). According to this hypothesis, Ca^{2+} binding to synaptotagmin 1 then triggers vesicle fusion by inducing the simultaneous binding of synaptotagmin 1 to SNARE complexes and to phospholipids, thereby displacing complexins and pulling the fusion pore open (see model in Figure 7). This hypothesis suggests that primed vesicles containing partly assembled SNARE complexes either are substrates for asynchronous Ca^{2+}-triggered release or are activated by complexin binding to SNARE complexes, which transforms them into a substrate for Ca^{2+}-triggered synchronous release. This hypothesis still includes a clamp function for complexins that, however, is secondary to their role in activating SNARE complexes and does not involve clamping of SNARE complexes as such, but only clamping of activated SNARE complexes produced by complexin binding in the first place.

The interplay between SNARE complexes, complexins, and synaptotagmin 1 is probably an evolutionarily old universal mechanism, as all organisms with a nervous system appear to express both synapotagmin-1 and complexin

(see for example Dykes and Davies, 2004). Moreover, this mechanism may be modulated by changing the affinity of either complexin or synaptotagmin 1 for SNARE complexes, as suggested by their phosphorylation (Hill et al., 2006), a modulation that could cause dramatic changes in the efficacy of synaptic transmission and remains to be evaluated.

EXPERIMENTAL PROCEDURES

Binding Assays

Recombinant proteins were purified as GST-fusion proteins with or without removal of GST (Sutton et al., 1998; Chen et al., 2002, 2006; Fernandez et al., 2001). Quantitative GST-affinity chromatography was performed with defined amounts of immobilized and soluble proteins, quantified after SDS-PAGE using Coomassie staining or quantitative immunoblotting, and analyzed with Prism software. For a detailed description of these and other methods, see the Supplemental Data.

Immunoprecipitations

Immunoprecipitations were performed with Triton X-100-solubilized rat brain proteins (McMahon et al., 1995) using polyclonal syntaxin 1 antibodies (U6250 or U6251) and polyclonal (P939) or monoclonal synaptobrevin 2 antibodies (Cl69.1). Immunoprecipitated proteins were quantified by immunoblotting using ^{125}I-labeled secondary antibodies and phosphorimager detection, and levels were normalized for the amount of immunoprecipitated syntaxin 1 or synaptobrevin 2. In competition experiments, controls showed that addition of 5 μM recombinant complexin did not significantly change the syntaxin or SNAP-25 amount in the immunoprecipitates (data not shown).

Experiments with Membrane-Reconstituted SNARE Complexes

SNARE complexes were assembled from purified full-length SNAP-25 and fragments from syntaxin 1A (residues 183–288) and synaptobrevin 2 (residues 1–96) in 1% β-octylglucoside and were reconstituted at a 1:1000 protein:lipid ratio into liposomes with a synaptic lipid composition (41% POPC, 32% DPPE, 12% DOPS, 5% PI, and 10% cholesterol [w/w]; Chen et al., 2006). Microfluidic channels (0.2 mm width, 0.2 mm height, 25 mm length) were formed using standard soft lithography techniques (Xia and Whitesides, 1998). Lanes of supported bilayers within microfluidic channels were formed with the vesicle fusion method (Brian and McConnell, 1984). A complexin 1 fragment (residues 26–83) with a V61C substitution was labeled with BODIPY-FL and imaged on a Leica confocal fluorescence microscope (TCS SP2). In a typical experiment, 50 nM BODIPY-FL-labeled complexin was incubated with a deposited supported bilayer for 15 min, unbound complexin was washed out, and unlabeled synaptotagmin 1 fragment (residues 140–421) was added in the presence of 1 mM EDTA or 1 mM Ca^{2+} and incubated with the bilayer for 10 min, followed by a wash with buffer containing either 1 mM EDTA or 1 mM Ca^{2+}, respectively. As a negative control, fluorescent complexin was added to bilayers lacking reconstituted SNAREs. Competition experiments were performed analogously (see Figure 3 legend). Data were analyzed with Image J (NIH) and Origin 6.0 programs and fitted to dose-response models.

Neuronal Cultures and Lentivirus Infection

Primary cortical neurons from E18 or P1 pups of wild-type rats or mice or from mice lacking synaptotagmin 1 or synaptobrevin 2 (Deak et al., 2004; Maximov and Südhof, 2005) were infected at 5 days in vitro with lentiviruses prepared in transfected HEK293T cells and analyzed at 14–16 days in vitro. For all experiments, expression of fusion proteins was confirmed by immunoblotting and Venus fluorescence. To visualize synaptic boutons, neurons were loaded with FM5-95 (400 μM; Molecular Probes) by a 90 s incubation in hyperkalemic solution containing (in mM) 45 KCl, 95 NaCl, 2 $MgCl_2$, 10 glucose, 10 HEPES, and 2 $CaCl_2$ and washed for 15 min in a normal bath solution containing (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES-NaOH [pH 7.4], and 10 glucose, and images were acquired in an Axiovert 200M confocal microscope (Carl Zeiss, Inc.).

Electrophysiology

Inhibitory postsynaptic currents (IPSCs) were evoked and analyzed as described (Maximov and Südhof, 2005). All statistical analyses were performed with Student's t test (*$p < 0.001$).

Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, and five figures and can be found with this article online at http://www.cell.com/cgi/content/full/126/6/1175/DC1/.

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