Neuron
Report

Doc2 Supports Spontaneous Synaptic Transmission by a Ca^{2+}-Independent Mechanism

Zhiping P. Pang,[1,3] Taulant Bacaj,[1,3] Xiaofei Yang,[1,3] Peng Zhou,[2] Wei Xu,[2] and Thomas C. Südhof[1,2,*]
[1]Department of Molecular and Cellular Physiology
[2]Howard Hughes Medical Institute
Stanford University, 265 Campus Drive, Stanford, CA 94305-5453, USA
[3]These authors contributed equally to this work
*Correspondence: tcs1@stanford.edu
DOI 10.1016/j.neuron.2011.03.011

SUMMARY

Two families of Ca^{2+}-binding proteins have been proposed as Ca^{2+} sensors for spontaneous release: synaptotagmins and Doc2s, with the intriguing possibility that Doc2s may represent high-affinity Ca^{2+} sensors that are activated by deletion of synaptotagmins, thereby accounting for the increased spontaneous release in synaptotagmin-deficient synapses. Here, we use an shRNA-dependent quadruple knockdown of all four Ca^{2+}-binding proteins of the Doc2 family to confirm that Doc2-deficient synapses exhibit a marked decrease in the frequency of spontaneous release events. Knockdown of Doc2s in synaptotagmin-1-deficient synapses, however, failed to reduce either the increased spontaneous release or the decreased evoked release of these synapses, suggesting that Doc2s do not constitute Ca^{2+} sensors for asynchronous release. Moreover, rescue experiments revealed that the decrease in spontaneous release induced by the Doc2 knockdown in wild-type synapses is fully reversed by mutant Doc2B lacking Ca^{2+}-binding sites. Thus, our data suggest that Doc2s are modulators of spontaneous synaptic transmission that act by a Ca^{2+}-independent mechanism.

INTRODUCTION

At a synapse, three forms of neurotransmitter release are observed: evoked synchronous, evoked asynchronous, and spontaneous "minirelease." Synchronous release is triggered by Ca^{2+}-binding to synaptotagmins and represents the dominant release mode, whereas asynchronous release is mediated by Ca^{2+}-binding to an as yet unknown Ca^{2+} sensor and becomes manifest only under certain conditions (Goda and Stevens, 1994; Maximov and Südhof, 2005; Sun et al., 2007; Kerr et al., 2008). Spontaneous release is also largely Ca^{2+} dependent (Li et al., 2009; Xu et al., 2009). Confusingly, two Ca^{2+} sensors were proposed to trigger spontaneous release in wild-type synapses: synaptotagmins, suggesting that spontaneous release is simply an extension of evoked synchronous release (Xu et al., 2009), and proteins of the Doc2 family, suggesting that spontaneous and evoked releases are governed by distinct Ca^{2+} sensors (Groffen et al., 2010).

Synaptotagmins and Doc2 proteins are similar in that both contain two homologous C_2 domains, but differ in that the former include an N-terminal transmembrane region, whereas the latter are cytosolic (Orita et al., 1995; Sakaguchi et al., 1995). Each protein family comprises Ca^{2+}-binding and Ca^{2+}-independent members (8 of 16 synaptotagmins bind Ca^{2+}, in particular the paradigmatic syntaptotagmin-1 [Syt1], while four Doc2-like proteins potentially bind Ca^{2+}, namely Doc2A, Doc2B, Doc2G, and rabphilin). The two protein families exhibit the same overall C_2 domain architecture, and display Ca^{2+}-dependent phospholipid- and SNARE-binding activities (Brose et al., 1992; Davletov and Südhof, 1993; Kojima et al., 1996; Groffen et al., 2006, 2010). Synaptotagmins perform a well-established function as Ca^{2+} sensors for exocytosis and Doc2 proteins were also shown to activate exocytosis (Orita et al., 1996; Mochida et al., 1998; Hori et al., 1999; Friedrich et al., 2008; Higashio et al., 2008). Consistent with a role for the Doc2 protein family in synaptic exocytosis, knockout (KO) studies suggested that rabphilin (which is closely related to Doc2s but includes an N-terminal zinc-finger domain absent from other members of this protein family; Fukuda, 2005) regulates repriming of vesicles for exocytosis (Deák et al., 2006). Strikingly, a recent double KO of Doc2A and Doc2B in neurons uncovered a large decrease in spontaneous release suggesting that Doc2s might act as Ca^{2+} sensors for spontaneous release (Groffen et al., 2010; Martens, 2010). Doc2 proteins are also interesting because the Doc2A gene is deleted or duplicated in 16p11.2 copy number variations associated with autism (Shinawi et al., 2010).

The notion that Doc2 proteins may act as Ca^{2+} sensors for spontaneous exocytosis was attractive given their biochemical properties, but surprising because synaptotagmins were previously shown to mediate most of the Ca^{2+} triggering of spontaneous release (Xu et al., 2009). Thus, the question arises how two Ca^{2+} sensors can mediate spontaneous release and whether one Ca^{2+} sensor is dominant over the other. Moreover, the continued expression of other similar Ca^{2+}-binding proteins (Doc2G and rabphilin) in the Doc2A/Doc2B double KO neurons prompts the question whether Doc2 proteins have additional functions that were occluded by the continued presence of these other Ca^{2+}-binding proteins.

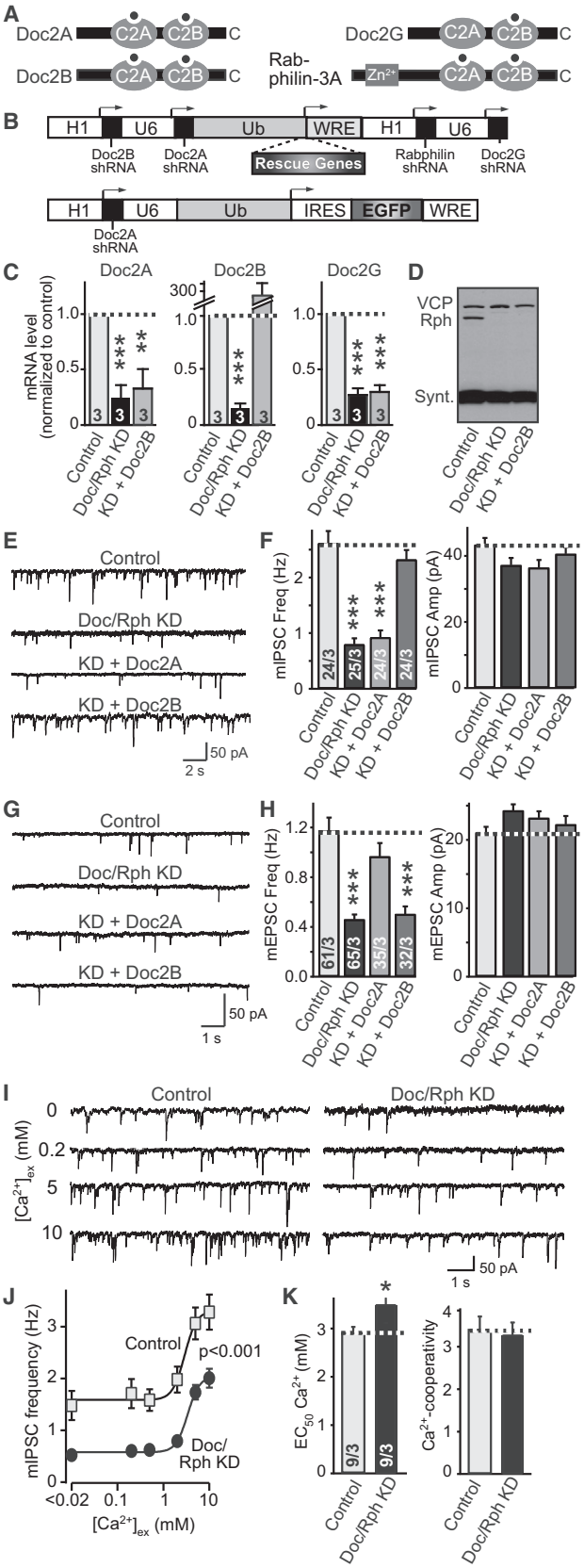

Figure 1. Knockdown of Doc2 Proteins Reduces Spontaneous Minirelease

(A) Domain structures of Doc2 proteins and rabphilin-3A. Note that rabphilin resembles a Doc2 protein with the characteristic C_2 domains but contains an extra N-terminal zinc-finger domain. Black dots = predicted Ca^{2+}-binding sites.

(B) Lentiviral system for KD of all four members of the Doc2 protein family. H1 and U6, human H1 and U6 pol III promoters; Ub, ubiquitin pol II promoter; WRE, woodchuck hepatitis virus regulatory element.

(C and D) Measurement of KD efficiency. Cortical neurons cultured from newborn mice were coinfected at DIV4 with a control lentivirus expressing only EGFP (control) with the two lentiviruses described in panel (B) either without a rescue cDNA (Doc/Rph KD) or with an shRNA-resistant rescue cDNA encoding Doc2B (KD + Doc2B). Cells were harvested at DIV14 and mRNA levels for the three Doc2 isoforms were measured by quantitative RT-PCR (C), whereas the protein levels for rabphilin were assessed by immunoblotting. Synt., syntaxin; VCP, vasolin-containing protein (loading controls).

(E and F) Representative traces (E) and summary graphs of the frequency (F, left) and amplitude (F, right) of inhibitory mIPSCs monitored in control neurons (control) and DR KD neurons without (Doc/Rph KD) or with expression of Doc2A or Doc2B rescue cDNA (KD + Doc2A or KD + Doc2B).

(G and H) Same as in panels (E) and (F) except that excitatory mEPSCs were recorded.

(I) Representative traces of mIPSCs monitored at different external Ca^{2+} concentrations in cortical neurons infected with control and DR KD lentiviruses.

(J) Plot of the mIPSC frequency as a function of the external Ca^{2+} concentration.

(K) Apparent Ca^{2+} affinity (left, estimated as the EC_{50} for the mIPSC frequency) and Ca^{2+} cooperativity (right) of spontaneous mIPSCs in control and DR KD neurons, calculated by Hill-function fits of individual Ca^{2+}-titration experiments. Data shown are means ± SEMs. In panel (C), n = 3 culture experiments. In (F), (H), and (K), the numbers of cells/experiments analyzed are shown in bars; n for (J) = (K). Statistical analyses for (F), (H), and (K) are by Student's t test (*$p < 0.05$ and ***$p < 0.001$), and for (J) are by two-way ANOVA. See also Figure S1.

To address these questions, we developed a lentiviral knockdown (KD) approach that allows quadruple RNAi experiments coupled with rescue controls. By using this approach, we examined synaptic transmission in neurons lacking all Ca^{2+}-binding members of the Doc2 family (Doc2A, Doc2B, Doc2G, and rabphilin). Our results confirm that suppression of Doc2 expression by the Doc2/rabphilin quadruple KD (referred to as DR KD) reduces spontaneous release dramatically (Groffen et al., 2010). However, Ca^{2+}-triggered asynchronous release is unimpaired in the KD neurons and the DR KD phenotype in spontaneous release was fully rescued by expression of a Ca^{2+}-binding-deficient mutant of Doc2B, suggesting that Doc2 functions in spontaneous release not as a Ca^{2+} sensor, but as a structural support element. Our data thus are consistent with the notion that for spontaneous release, synaptotagmins remain the primary Ca^{2+} sensors under normal conditions.

RESULTS

A Lentiviral RNAi System Targeting Four Different mRNAs

To overcome potential functional redundancy among Doc2 protein family members (Doc2A, Doc2B, Doc2G, and rabphilin; Figure 1A; Pang and Südhof, 2010), we designed a lentiviral RNAi strategy to suppress expression of all four Doc2-like proteins in cultured mouse cortical neurons. We first screened

for effective shRNAs that suppress each mRNA by at least 75% as measured by quantitative RT-PCR of mRNA levels and immunoblotting. We then generated a lentivirus capable of expressing all four effective shRNAs from pol III promoters (the human H1 and U6 promoters) and a rescue construct from a pol II promoter (the ubiquitin promoter; Figure 1B). Expression of the four shRNAs against Doc2 family proteins yielded good suppression of all targets except for Doc2A, although the KD efficiency was not as high as with lentiviruses expressing only a single shRNA. Thus, to maximize the Doc2A KD, we generated a second lentivirus expressing another Doc2A shRNA (Figure 1B) and superinfected the cultured cortical neurons with both viruses. This procedure produced ~75% KD of all four targets, allowing us to analyze the effects of such a loss-of-function manipulation (Figures 1C and 1D).

Doc2/Rabphilin KD Reduces Spontaneous Minirelease

Because Doc2B is a proposed Ca^{2+} sensor for spontaneous release (Groffen et al., 2010), we first tested the effect of the quadruple KD of Doc2A, Doc2B, Doc2C, and rabphilin (Doc2/rabphilin KD, or DR KD) on spontaneous miniature inhibitory and excitatory postsynaptic currents (mIPSCs and mEPSCs, respectively). Consistent with observations in Doc2A/Doc2B double KO mice (Groffen et al., 2010), we found that the DR KD reduced spontaneous inhibitory and excitatory minirelease by >60% (Figures 1E–1H) without altering neuronal cell density or synapse numbers and sizes (Figure S1A, available online).

With any shRNA-mediated KD, off-target effects are a major concern (Alvarez et al., 2006) even if the KD reproduces the KO phenotype (Groffen et al., 2010). To exclude off-target effects, we performed rescue experiments by coexpression of shRNA-resistant Doc2A or Doc2B alongside the shRNAs. Surprisingly, we found that Doc2A expression rescued the impairment of spontaneous minirelease in excitatory but not inhibitory synapses in DR KD neurons, whereas Doc2B conversely rescued the mIPSC but not the mEPSC phenotype (Figures 1E–1H).

To determine whether the DR KD acts postsynaptically, we transfected the lentiviral vectors resulting in the expression of the DR shRNAs and EGFP in only a few neurons. Electrophysiological recordings from transfected, fluorescent neurons detected no changes in mIPSC frequency (Figure S1C), suggesting a presynaptic role for Doc2 proteins.

Most spontaneous release is suppressed by BAPTA-AM, suggesting it is largely Ca^{2+} dependent (Li et al., 2009; Xu et al., 2009). To test whether the DR KD changes the Ca^{2+} dependence of spontaneous release, we titrated the extracellular Ca^{2+} dependence of the minifrequency. The DR KD decreased minirelease at all Ca^{2+} concentrations (Figures 1I and 1J) and produced a small increase in apparent Ca^{2+} affinity, but had no effect on apparent Ca^{2+} cooperativity (Figure 1K). Thus, the DR KD does not cause a major change in the Ca^{2+} dependence of minirelease, but primarily suppresses the amount of release.

DR KD Does Not Alter Evoked Synchronous or Asynchronous Release

Measurements of synaptic transmission evoked by isolated action potentials showed that the DR KD did not decrease evoked synchronous release (Figures 2A–2C), consistent with studies in Doc2A/Doc2B double KO mice (Groffen et al., 2010). Moreover, the DR KD did not alter the size of the readily releasable pool of vesicles as measured by application of hypertonic sucrose (Figures S2A and S2B).

Because Doc2 proteins may have a higher apparent Ca^{2+} affinity than synaptotagmins (Groffen et al., 2010; McMahon et al., 2010), it is possible that they act as Ca^{2+} sensors for asynchronous release. To explore this possibility, we first measured the effect of the DR KD on delayed release, a form of asynchronous release that can be assessed after a 10 Hz stimulus train (Maximov and Südhof, 2005). We observed a trend toward decreased delayed release (Figures 2D–2G). This trend, however, was not significant, prompting us to study asynchronous release further by using cortical neurons from Syt1 KO mice in which synchronous release is absent (Geppert et al., 1994). In these mice, spontaneous minirelease exhibits a paradoxical increase with a dramatically altered Ca^{2+} dependence (Xu et al., 2009) and delayed release is enhanced (Maximov and Südhof, 2005), suggesting that Syt1 functions not only as a Ca^{2+} sensor for spontaneous and evoked release, but also as a clamp for secondary Ca^{2+} sensors that mediate different forms of spontaneous and evoked release. Thus, we investigated the possibility that Doc2s represent secondary Ca^{2+} sensors that become activated in Syt1 KO neurons and may mediate these different forms of Ca^{2+}-triggered release.

We found that the DR KD had no significant effect on spontaneous minirelease in Syt1 KO neurons, suggesting that the DR KD effect on minirelease requires Syt1 and that Doc2s do not operate as the secondary Ca^{2+} sensors for the enhanced spontaneous release activated by the Syt1 KO (Figures 2H and 2I and Figures S2C–S2F). Because the high-minirelease rates in Syt1 KO neurons may saturate the response, we also measured the effect of the DR KD on minifrequency at a lower Ca^{2+} concentration (0.5 mM), but again failed to observe a change (Figures S2G and S2H). Moreover, we examined the effect of the DR KD on evoked asynchronous release in Syt1 KO neurons, but again did not detect an impairment (Figures 2J and 2K and Figures S2I and S2J). Thus, Doc2 proteins are not required for the increased spontaneous or asynchronous release in Syt1 KO neurons; the selective effect of the DR KD on spontaneous release in wild-type but not Syt1 KO synapses reinforces the notion that spontaneous release in these two preparations represents distinct processes.

Generation of Ca^{2+}-Binding Site Mutants of Doc2B

Based on sequence alignments (Figure S3) and the well-characterized Ca^{2+}-binding sites of the Syt1 and rabphilin C_2 domains (Chen et al., 2002; Ubach et al., 1998, 1999), the Doc2B C_2A and C_2B domains are predicted to bind two Ca^{2+} ions each (Figure 3A). To test the functional role of Ca^{2+}-binding to Doc2, we produced mutants of the Doc2B C_2 domains in which three of the five aspartate residues that ligate the Ca^{2+} ions have been exchanged for alanines (Figure S3), analogous to similar mutations that block Syt1 function (Shin et al., 2009). To ensure that the mutant C_2 domains still folded properly, we purified them as recombinant proteins and measured their circular dichroism spectra (Figures 3B and 3C). The wild-type and mutant C_2A

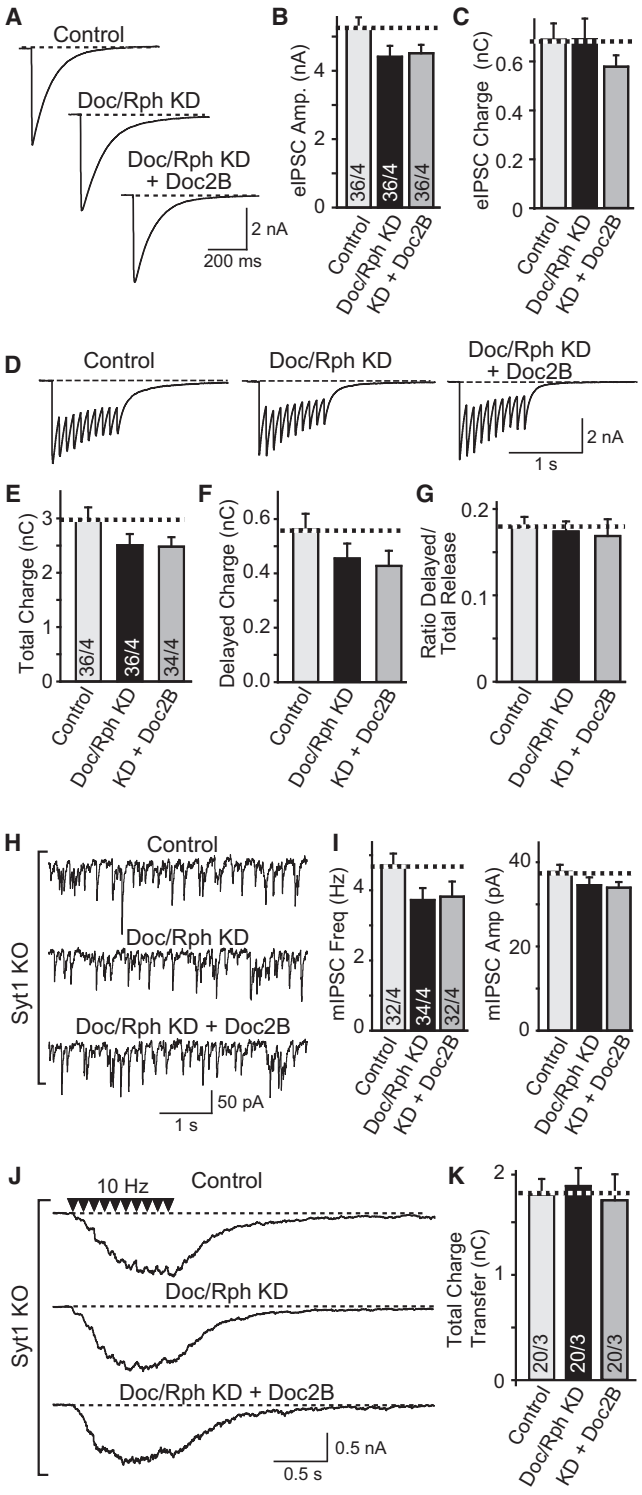

Figure 2. DR KD Does Not Change Evoked Synchronous or Asynchronous Release

(A–C) Representative traces (A) and summary graphs of the amplitude (B) and charge transfer (C) of IPSCs evoked by isolated action potentials in control neurons and DR KD neurons without or with Doc2B rescue. Neuronal KDs were performed as described for Figure 1C.

(D–G) Representative traces (D), total charge transfer (E), charge transfer during delayed release (F), and the ratio of delayed to total release measured by charge transfer (G) of IPSCs evoked by a 10 Hz stimulus train applied for 1 s.

(H and I) Representative traces (H) and the frequency (I, left) or amplitude (I, right) of mIPSCs monitored in cortical neurons cultured from Syt1 KO mice and infected with control lentivirus or the DR KD lentiviruses without or with Doc2B rescue as described in Figure 1C. For mEPSCs, see Figure S2.

(J and K) Representative traces (J) and total charge transfer (K) of IPSCs evoked by a 1 s 10 Hz stimulus train in Syt1 KO neurons infected with control or KD lentiviruses as above.

Data shown are means ± SEMs. Numbers of cells/experiments analyzed are shown in the bars. Student's t test failed to detect significant differences. See also Figure S2.

and C_2B domains exhibited similar characteristic β sheet spectra, indicating that they were well folded.

Because Ca^{2+}-binding to Doc2 C_2 domains has not been directly measured and it is uncertain whether Ca^{2+}-binding to these C_2 domains is blocked in the mutations we introduced, we examined Ca^{2+}-binding to the wild-type and mutant C_2B domain. In these measurements, we took advantage of a tryptophan residue adjacent to the predicted Ca^{2+}-binding site (W356) and monitored the intrinsic tryptophan fluorescence of the recombinant wild-type and mutant C_2B domain as a function of Ca^{2+} (Figure 3D). Similar to the C_2B domain of rabphilin (Ubach et al., 1999), addition of Ca^{2+} quenched the intrinsic tryptophan fluorescence of wild-type but not of mutant C_2B domain protein, demonstrating that the former but not the latter bound Ca^{2+}. Plots of the titrations suggested a low-micromolar-intrinsic Ca^{2+} affinity of the C_2B domain (Figure 3E). These results are consistent with indirect biochemical measurements, suggesting that Doc2 proteins exhibit a higher apparent Ca^{2+} affinity than Syt1 (Groffen et al., 2010). Note that we chose to target intrinsic Ca^{2+}-binding here instead of a secondary Ca^{2+}-dependent binding property of Doc2B, such as phospholipid binding, in order to ensure that the mutation would block all Ca^{2+}-dependent functions of Doc2B and not just one particular property.

Ca^{2+}-Binding to Doc2B Is Not Required for Rescuing Minirelease

In a final set of experiments, we tested whether rescue of the decrease in spontaneous release induced by the DR KD requires Ca^{2+}-binding to Doc2B. Surprisingly, mutant Doc2B in which all Ca^{2+}-binding sites were inactivated by mutations of the aspartate Ca^{2+} ligands in both C_2 domains fully reversed the >60% decrease in minifrequency induced by the DR KD (Figures 4A and 4B), suggesting that Doc2B acts in spontaneous release not as a Ca^{2+} sensor, but as a structural element supporting continued supply of vesicles for spontaneous exocytosis.

The unexpected rescue of the reduced minifrequency by mutant Doc2B in DR KD neurons could potentially be due to a shift in the Ca^{2+} dependence of spontaneous release, i.e., by activation of the secondary Ca^{2+} sensor that mediates spontaneous release in Syt1 KO synapses (Xu et al., 2009). To address this possibility, we titrated the Ca^{2+} dependence of minirelease in Doc2-deficient neurons without or with rescue with mutant Doc2B (Figures 4C and 4D). Strikingly, mutant Doc2B not only rescued minirelease at all Ca^{2+} concentrations, but even slightly enhanced it (Figure 4D) and reversed the small increase in apparent Ca^{2+} affinity

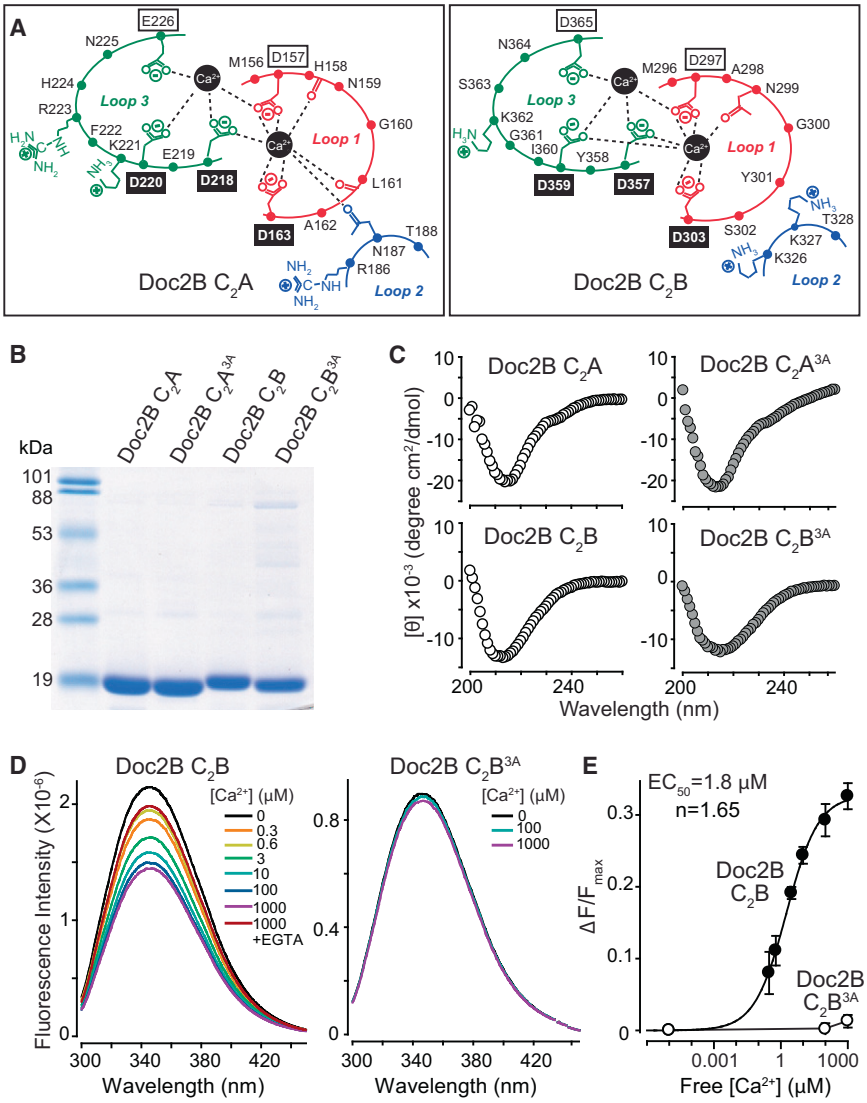

Figure 3. Ca^{2+}-Binding-Deficient Mutant C_2 Domains of Doc2B Are Folded

(A) Schema of the predicted Ca^{2+}-binding sites of the Doc2B C_2A (left) and Doc2B C_2B domain Ca^{2+}-binding sites (right), based on the atomic structures of the rabphilin C_2B domain and the Syt1 C_2A and C_2B domains (Fernández-Chacón et al., 2001; Fernandez et al., 2001; Ubach et al., 1999). Aspartate and glutamate residues involved in Ca^{2+}-binding are boxed; residues substituted for alanines in the Ca^{2+}-binding site mutants are shown on a black background.

(B) Purified wild-type or mutant Doc2B C_2 domains. The mutant Doc2B C_2A and C_2B domains contain three alanine substitutions each in critical Ca^{2+}-binding residues ($C2A^{3A}$: D163A, D218A, and D220A; $C2B^{3A}$: D303A, D357A, and D359A).

(C) Circular dichroism spectra of wild-type and mutant Doc2B C_2 domains.

(D) Ca^{2+} titration of intrinsic tryptophan fluorescence of wild-type and mutant Doc2B C_2B domains. Panel (D) depicts fluorescence spectra of the wild-type (left) and mutant Doc2 C_2B domain (right) as a function of increasing concentrations of free Ca^{2+}, followed by addition of excess EGTA (5 mM) to remove bound Ca^{2+}.

(E) A plot of the fluorescence changes ($\Delta F/F_{max}$) as a function of the free Ca^{2+} concentration on a semilogarithmic scale (note that 0 Ca^{2+} is plotted here at $\sim 10^{-5}$ μM for illustration purposes).

Data are representative experiments (C–E) or means ± SEMs (F, n = 3 independent experiments). See also Figure S3.

observed in the DR KD neurons (Figure 4E). Thus, mutant Doc2B is fully active in this functional assay.

DISCUSSION

Spontaneous minirelease probably mediates important information transfer and may be mechanistically distinct from evoked release (Sara et al., 2005; Fredj and Burrone, 2009; Stacey and Durand, 2000; Sutton et al., 2006). Most spontaneous release is Ca^{2+} dependent, and controlled by at least two different Ca^{2+} sensors: a low-affinity, high-cooperativity Ca^{2+} sensor in wild-type synapses and a high-affinity, low-cooperativity Ca^{2+} sensor in synaptotagmin- or complexin-deficient synapses (Sun et al., 2007; Xu et al., 2009; Yang et al., 2010). For wild-type synapses, two Ca^{2+} sensors for spontaneous release were proposed: synaptotagmins (Xu et al., 2009) and Doc2A and Doc2B (Groffen et al., 2010). No candidate Ca^{2+} sensor exists for minirelease in synaptotagmin-deficient synapses, although this Ca^{2+} sensor may be the same as that for asynchronous release, analogous to the proposed role of synaptotagmin as a Ca^{2+} sensor for both spontaneous and synchronous release in wild-type synapses. Both synaptotagmin and Doc2 are attractive Ca^{2+} sensor candidates for spontaneous release based on their biochemical properties, but only for synaptotagmin is there evidence linking changes in Ca^{2+}-binding affinity to changes in spontaneous release (Xu et al., 2009). Here, we have examined the potential role of Doc2 proteins as Ca^{2+} sensors in spontaneous release and their relation to asynchronous release. In doing so, we strove to avoid potential problems caused by the expression of four closely related isoforms of Doc2 proteins that could produce functional redundancy and developed an approach that allowed simultaneous KD of four different targets with a rescue control (Figures 1A and 1B).

Our data confirm KO studies showing that Doc2 proteins are essential for normal minirelease—in fact, the degree of impairment in spontaneous release we observed with a 75% KD of all four isoforms (Figure 1 and Figure S1) is strikingly similar to that described for the Doc2A and Doc2B double KO (Groffen et al., 2010). We show that in DR KD synapses, the apparent Ca^{2+} dependence of minirelease exhibits a small

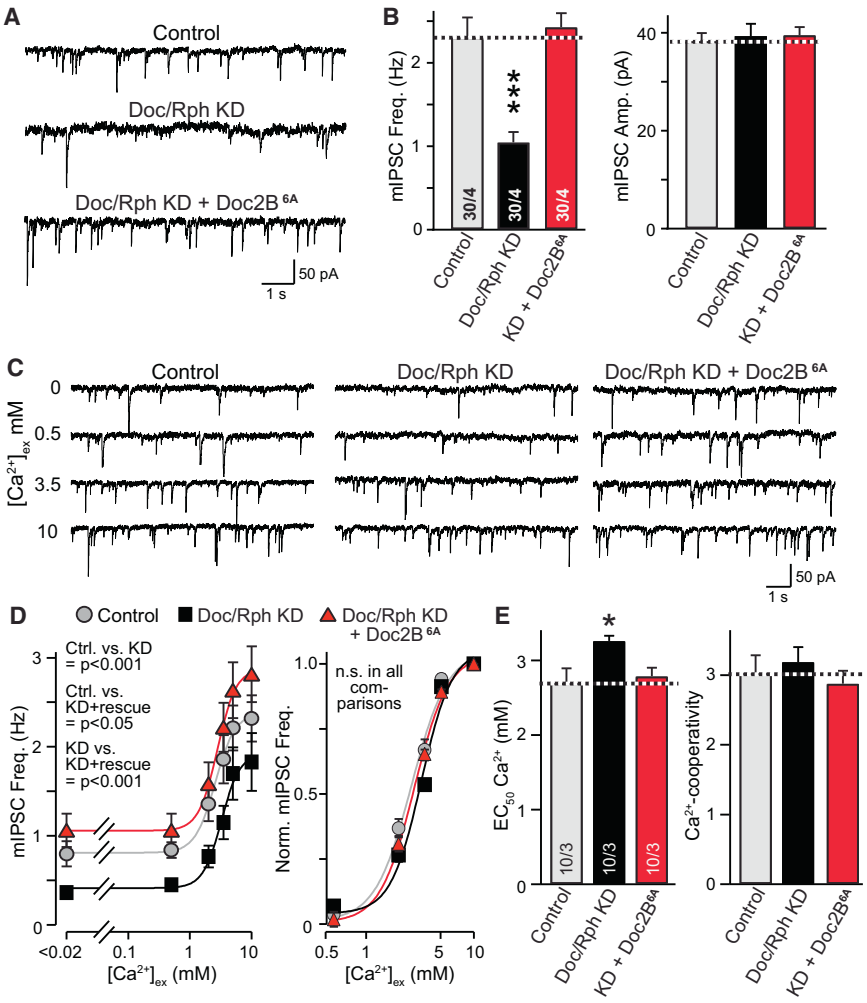

Figure 4. Ca^{2+}-Binding-Deficient Doc2B Rescues the Decrease in Miniature IPSC Frequency in DR KD Neurons

(A and B) Representative traces (A) and summary graphs of the frequency (B, left) and amplitude (B, right) of mIPSCs monitored in control neurons (control) and DR KD neurons without (Doc/Rph KD) or with expression of mutant Doc2B (KD + $Doc2B^{6A}$) in which all Ca^{2+}-binding sites were ablated (see Figure 3A).

(C) Representative traces of mIPSCs monitored at different external Ca^{2+} concentrations in cortical neurons infected with control lentivirus and DR KD lentiviruses without or with expression of mutant $Doc2B^{6A}$ rescue cDNA.

(D) Plot of the mean absolute (left) and normalized mIPSC frequency (right) as a function of the external Ca^{2+} concentration. mIPSCs were monitored in control infected neurons and DR KD neurons without and with rescue with mutant $Doc2B^{6A}$ as described in (C).

(E) Apparent Ca^{2+} affinity (left, estimated as the EC_{50} for the mIPSC frequency) and Ca^{2+} cooperativity (right) of spontaneous mIPSCs in control and DR KD neurons without or with rescue by mutant $Doc2B^{6A}$, as calculated from Hill-function fits of individual Ca^{2+}-titration experiments.

Data shown are means ± SEMs. Numbers of cells/experiments analyzed are shown in the bars. In (D), n = (E). Statistical analyses for (B) and (E) are by Student's t test (*p < 0.05 and ***p < 0.001), and for (D) are by two-way ANOVA.

but significant increase (Figure 1), but that otherwise no change in Ca^{2+} triggering of either spontaneous or evoked release is detected (Figure 2). Moreover, our results indicate that the DR KD does not alter synchronous or asynchronous evoked release and—importantly—does not impair the enhanced spontaneous release detected in Syt1 KO synapses (Figure 2). This latter result confirms the notion that spontaneous release events in Syt1 KO and wild-type neurons are qualitatively different, consistent with their distinct Ca^{2+} dependence (Xu et al., 2009).

To test the role of Ca^{2+}-binding to Doc2 in spontaneous release, we generated Doc2 mutants unable to bind Ca^{2+} (Figure 3). Rescue experiments surprisingly revealed that mutant Doc2B lacking functional Ca^{2+}-binding sites was fully capable of rescuing the decrease in minifrequency induced by the DR KD and also rescued the altered apparent Ca^{2+} affinity of minirelease (Figure 4). Thus, Doc2 is unlikely to function as a Ca^{2+} sensor for minirelease, but rather acts in a structural, Ca^{2+}-independent role to maintain spontaneous minirelease consistent with a special status of spontaneous release (Sara et al., 2005; Fredj and Burrone, 2009).

Our results appear to contradict those of Groffen et al. (2010) who did not use mutations blocking Ca^{2+}-binding to Doc2B to test its role in minirelease, but other point mutations that supported a Ca^{2+} sensor role for Doc2B in minirelease. However, this apparent contradiction can be explained if one considers our current understanding of C_2 domains. Groffen et al. (2010) examined a gain-of-function mutation in the Ca^{2+}-binding mutations of the Doc2B C_2A domain that was modeled after a similar mutation in Syt1 (Pang et al., 2006; Stevens and Sullivan, 2003) and was also independently tested for Doc2B in chromaffin cells (Friedrich et al., 2008). The fact that this mutation increases minirelease in synapses does not necessarily mean that Doc2B is a direct Ca^{2+} sensor for release, but could equally change its structural role in minirelease especially because no correlation of a change in Ca^{2+} affinity of Doc2B with that of minirelease, as documented for Syt1 (Xu et al., 2009), was reported. Thus, it seems likely that Doc2 proteins are evolutionarily novel effectors for spontaneous minirelease which may have additional, as yet uncharacterized Ca^{2+}-dependent functions.

EXPERIMENTAL PROCEDURES

Generation of RNAi vectors

All shRNA expression, with and without rescue, was performed with the same lentiviral vector system (Pang et al., 2010; see Figure 1B for the schematic

diagram of vectors). Oligonucleotide sequences are described in Supplemental Experimental Procedures.

Production of Recombinant Lentiviruses

Production of recombinant lentiviruses was achieved by transfection of HEK293T cells with FuGENE-6 (Roche) as described (Pang et al., 2010; see Supplemental Experimental Procedures).

Neuronal Cultures and Immunocytochemistry

Cortical neurons were cultured from neonatal wild-type or Syt1 KO mice as described (Pang et al., 2010), infected at 5 days in vitro (DIV5), and analyzed at DIV14–16 (see Supplemental Experimental Procedures for detailed descriptions).

Electrophysiological Recordings

Electrophysiological recordings were performed by using whole-cell recordings and concentric extracellular stimulation electrodes (Maximov et al., 2007; see Supplemental Experimental Procedures).

Purification and Biophysical Analyses

Purification and biophysical analyses of recombinant proteins were performed as described in the Supplemental Experimental Procedures.

Miscellaneous

Immunocytochemistry and immunoblotting were performed as described (Chubykin et al., 2007).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.03.011.

ACKNOWLEDGMENTS

We thank Ira Huryeva for excellent technical support and Dr. Axel Brunger for help with circular dichroism spectra measurements. This paper was supported by a Recovery Act grant from the National Institute of Mental Health ([NIMH] 1R01 MH089054), a NIMH Conte Center Award (P50 MH086403), a NARSAD Young Investigator Award (to Z.P.P.), and a National Institute of Neurological Disorders and Stroke National Research Service Award fellowship (1F32NS067896, to T.B.).

Accepted: February 8, 2011
Published: April 27, 2011

REFERENCES

Alvarez, V.A., Ridenour, D.A., and Sabatini, B.L. (2006). Retraction of synapses and dendritic spines induced by off-target effects of RNA interference. J. Neurosci. *26*, 7820–7825.

Brose, N., Petrenko, A.G., Südhof, T.C., and Jahn, R. (1992). Synaptotagmin: A calcium sensor on the synaptic vesicle surface. Science *256*, 1021–1025.

Chen, X., Tomchick, D.R., Kovrigin, E., Araç, D., Machius, M., Südhof, T.C., and Rizo, J. (2002). Three-dimensional structure of the complexin/SNARE complex. Neuron *33*, 397–409.

Chubykin, A.A., Atasoy, D., Etherton, M.R., Brose, N., Kavalali, E.T., Gibson, J.R., and Südhof, T.C. (2007). Activity-dependent validation of excitatory versus inhibitory synapses by neuroligin-1 versus neuroligin-2. Neuron *54*, 919–931.

Davletov, B.A., and Südhof, T.C. (1993). A single C2 domain from synaptotagmin I is sufficient for high affinity Ca^{2+}/phospholipid binding. J. Biol. Chem. *268*, 26386–26390.

Deák, F., Shin, O.H., Tang, J., Hanson, P., Ubach, J., Jahn, R., Rizo, J., Kavalali, E.T., and Südhof, T.C. (2006). Rabphilin regulates SNARE-dependent re-priming of synaptic vesicles for fusion. EMBO J. *25*, 2856–2866.

Fernandez, I., Araç, D., Ubach, J., Gerber, S.H., Shin, O., Gao, Y., Anderson, R.G., Südhof, T.C., and Rizo, J. (2001). Three-dimensional structure of the synaptotagmin 1 C2B-domain: Synaptotagmin 1 as a phospholipid binding machine. Neuron *32*, 1057–1069.

Fernández-Chacón, R., Königstorfer, A., Gerber, S.H., García, J., Matos, M.F., Stevens, C.F., Brose, N., Rizo, J., Rosenmund, C., and Südhof, T.C. (2001). Synaptotagmin I functions as a calcium regulator of release probability. Nature *410*, 41–49.

Fredj, N.B., and Burrone, J. (2009). A resting pool of vesicles is responsible for spontaneous vesicle fusion at the synapse. Nat. Neurosci. *12*, 751–758.

Friedrich, R., Groffen, A.J., Connell, E., van Weering, J.R., Gutman, O., Henis, Y.I., Davletov, B., and Ashery, U. (2008). DOC2B acts as a calcium switch and enhances vesicle fusion. J. Neurosci. *28*, 6794–6806.

Fukuda, M. (2005). Versatile role of Rab27 in membrane trafficking: Focus on the Rab27 effector families. J. Biochem. *137*, 9–16.

Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F., and Südhof, T.C. (1994). Synaptotagmin I: A major Ca^{2+} sensor for transmitter release at a central synapse. Cell *79*, 717–727.

Goda, Y., and Stevens, C.F. (1994). Two components of transmitter release at a central synapse. Proc. Natl. Acad. Sci. USA *91*, 12942–12946.

Groffen, A.J., Friedrich, R., Brian, E.C., Ashery, U., and Verhage, M. (2006). DOC2A and DOC2B are sensors for neuronal activity with unique calcium-dependent and kinetic properties. J. Neurochem. *97*, 818–833.

Groffen, A.J., Martens, S., Díez Arazola, R., Cornelisse, L.N., Lozovaya, N., de Jong, A.P., Goriounova, N.A., Habets, R.L., Takai, Y., Borst, J.G., et al. (2010). Doc2b is a high-affinity Ca2+ sensor for spontaneous neurotransmitter release. Science *327*, 1614–1618.

Higashio, H., Nishimura, N., Ishizaki, H., Miyoshi, J., Orita, S., Sakane, A., and Sasaki, T. (2008). Doc2 α and Munc13-4 regulate $Ca^{(2+)}$-dependent secretory lysosome exocytosis in mast cells. J. Immunol. *180*, 4774–4784.

Hori, T., Takai, Y., and Takahashi, T. (1999). Presynaptic mechanism for phorbol ester-induced synaptic potentiation. J. Neurosci. *19*, 7262–7267.

Kerr, A.M., Reisinger, E., and Jonas, P. (2008). Differential dependence of phasic transmitter release on synaptotagmin 1 at GABAergic and glutamatergic hippocampal synapses. Proc. Natl. Acad. Sci. USA *105*, 15581–15586.

Kojima, T., Fukuda, M., Aruga, J., and Mikoshiba, K. (1996). Calcium-dependent phospholipid binding to the C2A domain of a ubiquitous form of double C2 protein (Doc2 beta). J. Biochem. *120*, 671–676.

Li, G.L., Keen, E., Andor-Ardó, D., Hudspeth, A.J., and von Gersdorff, H. (2009). The unitary event underlying multiquantal EPSCs at a hair cell's ribbon synapse. J. Neurosci. *29*, 7558–7568.

Martens, S. (2010). Role of C2 domain proteins during synaptic vesicle exocytosis. Biochem. Soc. Trans. *38*, 213–216.

Maximov, A., and Südhof, T.C. (2005). Autonomous function of synaptotagmin 1 in triggering synchronous release independent of asynchronous release. Neuron *48*, 547–554.

Maximov, A., Pang, Z.P., Tervo, D.G., and Südhof, T.C. (2007). Monitoring synaptic transmission in primary neuronal cultures using local extracellular stimulation. J. Neurosci. Methods *161*, 75–87.

McMahon, H.T., Kozlov, M.M., and Martens, S. (2010). Membrane curvature in synaptic vesicle fusion and beyond. Cell *140*, 601–605.

Mochida, S., Orita, S., Sakaguchi, G., Sasaki, T., and Takai, Y. (1998). Role of the Doc2 alpha-Munc13-1 interaction in the neurotransmitter release process. Proc. Natl. Acad. Sci. USA *95*, 11418–11422.

Orita, S., Sasaki, T., Naito, A., Komuro, R., Ohtsuka, T., Maeda, M., Suzuki, H., Igarashi, H., and Takai, Y. (1995). Doc2: A novel brain protein having two repeated C2-like domains. Biochem. Biophys. Res. Commun. *206*, 439–448.

Orita, S., Sasaki, T., Komuro, R., Sakaguchi, G., Maeda, M., Igarashi, H., and Takai, Y. (1996). Doc2 enhances Ca2+-dependent exocytosis from PC12 cells. J. Biol. Chem. *271*, 7257–7260.

Pang, Z.P., and Südhof, T.C. (2010). Cell biology of Ca2+-triggered exocytosis. Curr. Opin. Cell Biol. *22*, 496–505.

Pang, Z.P., Shin, O.H., Meyer, A.C., Rosenmund, C., and Südhof, T.C. (2006). A gain-of-function mutation in synaptotagmin-1 reveals a critical role of Ca2+-dependent soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex binding in synaptic exocytosis. J. Neurosci. *26*, 12556–12565.

Pang, Z.P., Xu, W., Cao, P., and Südhof, T.C. (2010). Calmodulin controls synaptic strength via presynaptic activation of CaM kinase II. J. Neurosci. *30*, 4132–4142.

Sakaguchi, G., Orita, S., Maeda, M., Igarashi, H., and Takai, Y. (1995). Molecular cloning of an isoform of Doc2 having two C2-like domains. Biochem. Biophys. Res. Commun. *217*, 1053–1061.

Sara, Y., Virmani, T., Deák, F., Liu, X., and Kavalali, E.T. (2005). An isolated pool of vesicles recycles at rest and drives spontaneous neurotransmission. Neuron *45*, 563–573.

Shin, O.H., Xu, J., Rizo, J., and Südhof, T.C. (2009). Differential but convergent functions of Ca^{2+} binding to synaptotagmin-1 C2 domains mediate neurotransmitter release. Proc. Natl. Acad. Sci. USA *106*, 16469–16474.

Shinawi, M., Liu, P., Kang, S.H., Shen, J., Belmont, J.W., Scott, D.A., Probst, F.J., Craigen, W.J., Graham, B.H., Pursley, A., et al. (2010). Recurrent reciprocal 16p11.2 rearrangements associated with global developmental delay, behavioural problems, dysmorphism, epilepsy, and abnormal head size. J. Med. Genet. *47*, 332–341.

Stacey, W.C., and Durand, D.M. (2000). Stochastic resonance improves signal detection in hippocampal CA1 neurons. J. Neurophysiol. *83*, 1394–1402.

Stevens, C.F., and Sullivan, J.M. (2003). The synaptotagmin C2A domain is part of the calcium sensor controlling fast synaptic transmission. Neuron *39*, 299–308.

Sun, J., Pang, Z.P., Qin, D., Fahim, A.T., Adachi, R., and Südhof, T.C. (2007). A dual-Ca2+-sensor model for neurotransmitter release in a central synapse. Nature *450*, 676–682.

Sutton, M.A., Ito, H.T., Cressy, P., Kempf, C., Woo, J.C., and Schuman, E.M. (2006). Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. Cell *125*, 785–799.

Ubach, J., Zhang, X., Shao, X., Südhof, T.C., and Rizo, J. (1998). Ca2+ binding to synaptotagmin: How many Ca2+ ions bind to the tip of a C2-domain? EMBO J. *17*, 3921–3930.

Ubach, J., García, J., Nittler, M.P., Südhof, T.C., and Rizo, J. (1999). Structure of the Janus-faced C2B domain of rabphilin. Nat. Cell Biol. *1*, 106–112.

Xu, J., Pang, Z.P., Shin, O.H., and Südhof, T.C. (2009). Synaptotagmin-1 functions as a Ca2+ sensor for spontaneous release. Nat. Neurosci. *12*, 759–766.

Yang, X.F., Kaeser-Woo, Y.J., Pang, Z.P., Xu, W., and Südhof, T.C. (2010). Complexin clamps asynchronous release by blocking a secondary Ca^{2+} sensor via its accessory α helix. Neuron *68*, 907–920.