Cellular/Molecular

C-Terminal Complexin Sequence Is Selectively Required for Clamping and Priming But Not for Ca^{2+} Triggering of Synaptic Exocytosis

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Complexins are small soluble proteins that bind to assembling SNARE complexes during synaptic vesicle exocytosis, which in turn mediates neurotransmitter release. Complexins are required for clamping of spontaneous "mini" release and for the priming and synaptotagmin-dependent Ca^{2+} triggering of evoked release. Mammalian genomes encode four complexins that are composed of an N-terminal unstructured sequence that activates synaptic exocytosis, an accessory α-helix that clamps exocytosis, an essential central α-helix that binds to assembling SNARE complexes and is required for all of its functions, and a long, apparently unstructured C-terminal sequence whose function remains unclear. Here, we used cultured mouse neurons to show that the C-terminal sequence of complexin-1 is not required for its synaptotagmin-activating function but is essential for its priming and clamping functions. Wild-type complexin-3 did not clamp exocytosis but nevertheless fully primed and activated exocytosis. Strikingly, exchanging the complexin-1 C terminus for the complexin-3 C terminus abrogated clamping, whereas exchanging the complexin-3 C terminus for the complexin-1 C terminus enabled clamping. Analysis of point mutations in the complexin-1 C terminus identified two single amino-acid substitutions that impaired clamping without altering the activation function of complexin-1. Examination of release induced by stimulus trains revealed that clamping-deficient C-terminal complexin mutants produced a modest relative increase in delayed release. Overall, our results show that the relatively large C-terminal complexin-1 sequence acts in priming and clamping synaptic exocytosis and demonstrate that the clamping function is not conserved in complexin-3, presumably because of its distinct C-terminal sequences.

Introduction

Synaptic vesicle fusion, like all intracellular fusion reactions except for mitochondrial fusion, is thought to be mediated by the assembly of SNARE complexes and the action of SM proteins (Sec1/Munc18-like) on these SNARE complexes (for review, see Südhof and Rothman, 2009). SNARE and SM proteins are controlled by synaptotagmins as Ca^{2+} sensors that trigger fusion-pore opening when Ca^{2+} binds to their C2 domains and induces binding of these C2 domains to phospholipids and SNARE complexes (Geppert et al., 1994; Fernández-Chacón et al., 2001) and by complexins, which are small soluble adaptor proteins that bind to SNARE complexes (McMahon et al., 1995).

A large number of studies of complexin function with different approaches has uncovered three principal functions for complexin in fusion: activation of SNARE complexes for subsequent Ca^{2+} triggering by synaptotagmin (Reim et al., 2001; Tang et al., 2006; Xue et al., 2007, 2009, 2010; Maximov et al., 2009; Strenzke et al., 2009); clamping of SNARE complexes preventing fusion (Giraudo et al., 2006, 2008, 2009; Schaub et al., 2006; Tang et al., 2006; Huntwork and Littleton, 2007; Yoon et al., 2008; Cho et al., 2010; Yang et al., 2010; Hobson et al., 2011; Martin et al., 2011); and priming of vesicles for fusion (Cai et al., 2008; Xue et al., 2010; Yang et al., 2010).

Mammals express four complexins, of which complexin-1 and complexin-2 are present in all neuronal and non-neuronal cells (McMahon et al., 1995), whereas complexin-3 and complexin-4 are expressed at high levels in retina (Reim et al., 2005). Crystal structures of complexin bound to SNARE complexes revealed that complexin contains unstructured N-terminal and C-terminal sequences (residues 1–22 and 71-134, respectively, in mouse complexin-1) that flank two α-helices, namely a more N-terminal accessory α-helix (residues 23–47) and a central α-helix (residues 48–70; Bracher et al., 2002; Chen et al., 2002). The N-terminal complexin region is essential for activating fusion (Xue et al., 2007; Maximov et al., 2009) and for vesicle priming (Yang et al., 2010), the accessory α-helix is essential for clamping fusion (Maximov et al., 2009), and the central α-helix of complexin binds to the SNARE complex and is necessary for all complexin functions (Tang et al., 2006; Maximov et al., 2009; Martin et al., 2011). However, the functions of the C-terminal region of complexins that accounts for almost half of their sequences remain unclear. Initial experi-

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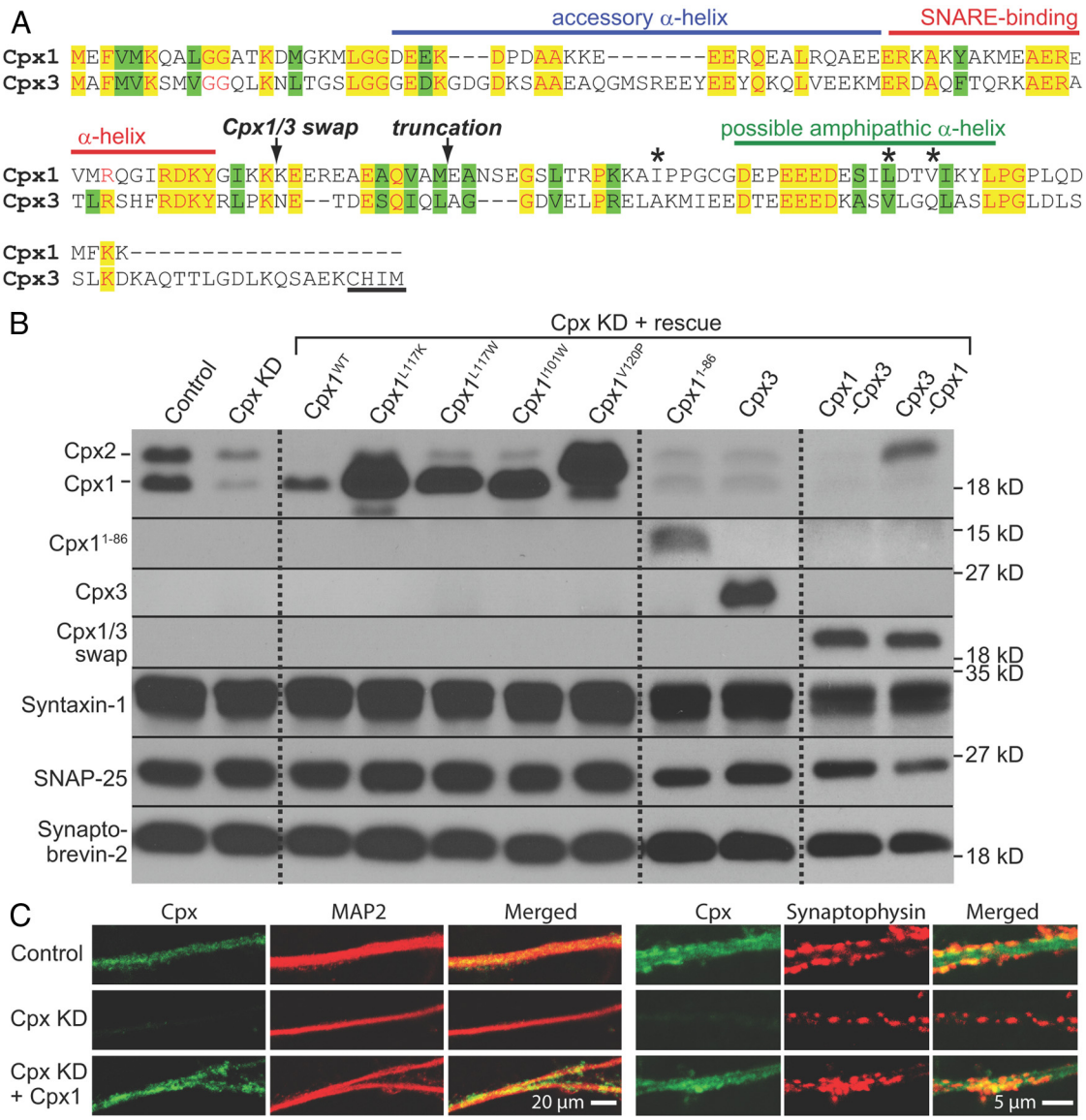

Figure 1. Comparison of complexin-1 and complexin-3 sequences and analysis of the C-terminal complexin-1 sequence in clamping spontaneous mini exocytosis. ***A***, Alignment of the mouse complexin-1 and complexin-3 sequences. Identical residues are highlighted in yellow and similar residues in green. The positions of the accessory and the SNARE-binding central α-helices are marked in blue and red, respectively, and the C-terminal isoprenylation sequence in complexin-3 is underlined. The positions in the complexin sequences at which the complexin-1 and complexin-3 sequences are swapped to generate complexin-1/3 hybrids (see Fig. 4) or where complexin-1 was truncated are marked by arrows, and the residues that were subjected to point mutations (see Fig. 7) are identified by asterisks. ***B***, Immunoblots of cultured neurons that were infected either with control lentivirus (Control), or with lentivirus expressing the complexin-1/2 shRNA alone (Cpx KD) or together with various complexin rescue proteins as indicated [Cpx1^{WT}, wild-type complexin-1; various complexin-1 point mutants (Cpx1^{L117K}; Cpx1^{L117W}; Cpx1^{I101W}; Cpx1^{V120P}); Cpx1$^{1-86}$, C-terminally truncated complexin-1; Cpx3, wild-type complexin-3; Cpx1–Cpx3 and Cpx3–Cpx1, complexin-1/3 and complexin-3/1 hybrid proteins; see *A*]. Neurons were infected at DIV5 and analyzed at DIV14. Although the blots indicate robust expression of all complexin proteins analyzed, expression levels cannot be directly compared between proteins because different complexin proteins exhibit distinct antibody reactivities. The top complexin blot was probed with the complexin antibody P942 (see Materials and Methods), the blot analyzing truncated complexin-1$^{1-86}$ was probed with antibody L669, and the complexin-3 and complexin-1/3 swap mutants were probed with antibody 122 301. Cpx3–Cpx1 was detected both with antibodies P942 and 122 301. ***C***, Representative images of cultured cortical neurons infected with control lentivirus or with lentivirus expressing the complexin shRNA plus either GFP or wild-type complexin-1 (Cpx1). Neurons were infected at DIV4, fixed at DIV14, and stained by double-immunofluorescence labeling with the polyclonal complexin antibody P942 and monoclonal antibodies to either MAP2 (left) or synaptophysin (right) antibodies. Scale bar in the right bottom corner applies to all images in a set.

ments suggested that this sequence is functionally inert (Xue et al., 2007) or that it may participate in Ca^{2+} triggering of fusion by binding to synaptotagmin-1 (Tokumaru et al., 2008) or to phospholipids (Malsam et al., 2009; Seiler et al., 2009). Recently, moreover, it was proposed in *Caenorhabditis elegans* that this sequence may clamp fusion (Martin et al., 2011). Thus, to explore the functional significance of C-terminal domain of complexins, we here performed structure–function experiments using complexin knockdown (KD) neurons and tested different forms and mutations of complexins. We find that the complexin C-terminal sequences are required for both clamping spontaneous fusion and for priming evoked fusion but are dispensable for activating synaptagmin-dependent Ca^{2+} triggering of fusion.

Materials and Methods

Generation of KD lentiviruses and rescue constructs. Complexin KD, wild-type complexin (Cpx^{WT}) rescue, and C-terminally truncated complexin-1 (Cpx1$^{1-86}$) rescue constructs were described previously

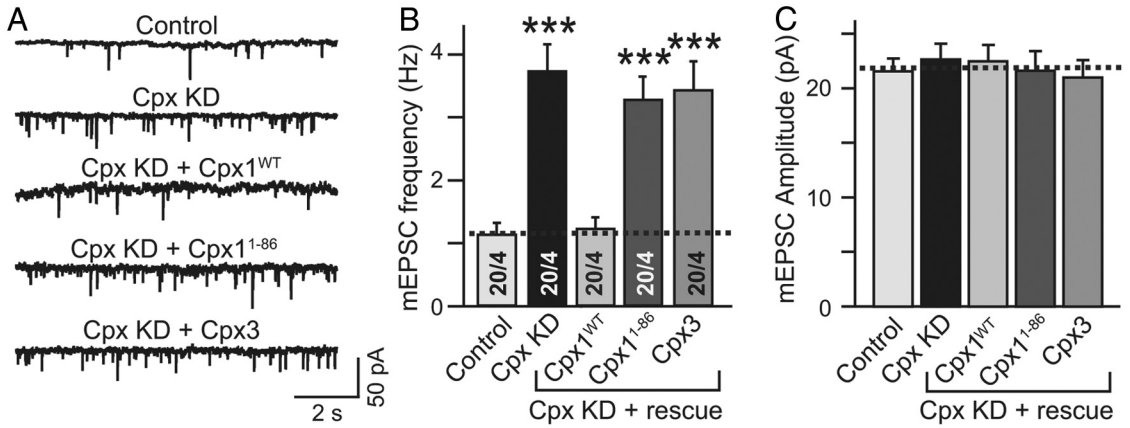

Figure 2. The C-terminal complexin-1 sequence is essential for clamping spontaneous mini exocytosis. ***A***, Representative traces of spontaneous mEPSCs recorded in the presence of tetrodotoxin (1 μM) in cultured cortical neurons that were infected with either control lentivirus (Control) or with complexin KD lentivirus expressing either no rescue construct (Cpx KD) or the indicated complexin constructs (Cpx1^{WT}, wild-type complexin-1; Cpx1$^{1-86}$, C-terminally truncated complexin-1; Cpx3, wild-type complexin-3). ***B***, ***C***, Summary graphs of the frequency (***B***) and amplitude (***C***) of spontaneous mEPSCs. Data are means ± SEMs; number of cells/independent cultures analyzed are depicted in the bars. Statistical significance was analyzed by Student's *t* test comparing the various complexin KD conditions with the control (***$p < 0.001$).

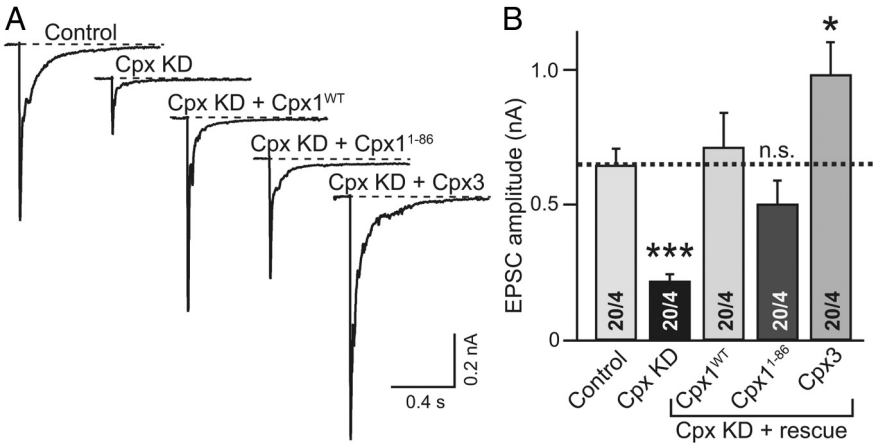

Figure 3. Analysis of the activity of the C-terminal complexin-1 sequence and of complexin-3 in the Ca^{2+} triggering of synaptic exocytosis. ***A***, ***B***, Sample traces of action-potential-evoked EPSCs (***A***) and summary graph of EPSC amplitudes (***B***) recorded in cultured cortical neurons that were infected with either control lentivirus (Control) or the complexin-1/2 KD lentivirus that expresses either no rescue construct or the indicated complexin constructs (Cpx1^{WT}, wild-type complexin-1; Cpx1$^{1-86}$, C-terminally truncated complexin-1; Cpx3, wild-type complexin-3). Data in *B* are means ± SEMs; number of cells/independent cultures analyzed are depicted in the bars. Statistical significance was analyzed by Student's *t* test comparing complexin KD neurons with control neurons (*$p < 0.05$; ***$p < 0.001$).

(Maximov et al., 2009; Yang et al., 2010). Cpx^{I101W}, Cpx^{L117W}, Cpx^{L117K}, and Cpx^{V120P} mutations were generated by gene synthesis and were cloned into the downstream of human ubiquitin promoter of L309 lentiviral vector (Pang et al., 2010).

Neuronal cultures. Cortical neurons were cultured from newborn wild-type mice of either sex as described previously (Maximov et al., 2009). Briefly, mouse cortices were dissected from postnatal day 1 mice, dissociated by papain digestion (10 U/ml, with 1 μM Ca^{2+} and 0.5 μM EDTA) for 20 min at 37°C, and plated on Matrigel-coated circular glass coverslips (11 mm diameter). Neurons were maintained for 14–16 d in MEM (Invitrogen) supplemented with B27 (Invitrogen), glucose, transferrin, fetal bovine serum, and AraC (Sigma).

Lentiviral production and infection of neuronal culture. Cpx KD lentiviruses were prepared as described previously (Maximov et al., 2009; Yang et al., 2010). The lentiviral expression vector (complexin-1/2 KD vectors and/or the complexin rescue vectors) and three helper plasmids (pRSV–REV, pMDLg/pRRE, and VSV-G) were cotransfected with the lentiviral vectors into human embryonic kidney 293T (HEK293T) cells (American Type Culture Collection) at 4, 2, 2, and 2 μg of DNA, respectively, per 25 cm^2 culture area using FUGENE6 transfection reagent (Roche). At 48 h after transfection, HEK cell medium was collected and clarified by centrifugation (500 × *g* for 5 min), and the supernatant was added directly to the medium of cultured cortical neurons maintained in 24-well plates (200 μl of supernatant per well). Cultured cortical neurons were infected at 4–5 d *in vitro* (DIV4–DIV5) and used for physiological analysis on DIV14–DIV16. In all experiments, the infection efficiency of neurons was monitored by the GFP signal driven by the ubiquitin promoter in the lentiviral vectors. All steps were performed under biosafety level 2 conditions. Quantitative RT-PCR indicated that the KD efficiency was 70–80% (Maximov et al., 2009).

Immunoblotting. Cultured neurons infected with the control or complexin KD lentiviruses were washed twice with PBS at DIV14, lysed in SDS–lysate buffer (25 mM HEPES-NaOH, pH 7.2, 25 mM NaCl, and 4 mM EDTA; 25 μl of sample buffer per well for 24-well plates), and boiled for 20 min. Equivalent amounts of protein (20 μl) were analyzed by SDS-PAGE and immunoblotting using the following antibodies: rabbit polyclonal antibody against the C terminus of Cpx1 (P942), rabbit polyclonal antibody against full-length GST-Cpx1 (L669), complexin-3 (122 301; Synaptic Systems), syntaxin-1 (78.2; Synaptic Systems), SNAP-25 (71.1; Synaptic Systems), and synaptobrevin-2 (69.1; Synaptic Systems).

Immunocytochemistry of cultured neurons was performed essentially as described previously (McMahon et al., 1995; Tang et al., 2006).

Electrophysiology. Whole-cell patch-clamp recordings were made with neurons at DIV14–DIV16 with a pipette solution containing the following (in mM): 135 CsCl, 5 NaCl, 1 $MgCl_2$ 10 HEPES-NaOH, pH 7.4, 10 EGTA, 4 Mg-ATP, 0.4 Na-GTP, and 5 QX-314 (lidocaine *N*-ethyl bromide). The bath solution contained the following (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES-NaOH, pH 7.4, and 10 glucose. AMPA-receptor-mediated EPSCs (recorded at a holding potential of −70 mV) were isolated pharmacologically with 50 μM D-APV and 100 μM picrotoxin in the bath solution, and NMDA-receptor-mediated EPSCs (recorded at a holding potential of +40 mV) were isolated with 20 μM CNQX, 100 μM picrotoxin, and 15 μM glycine and by raising the $MgCl_2$ concentration to 4 mM. mEPSCs was recorded in 1 μM tetrodo-

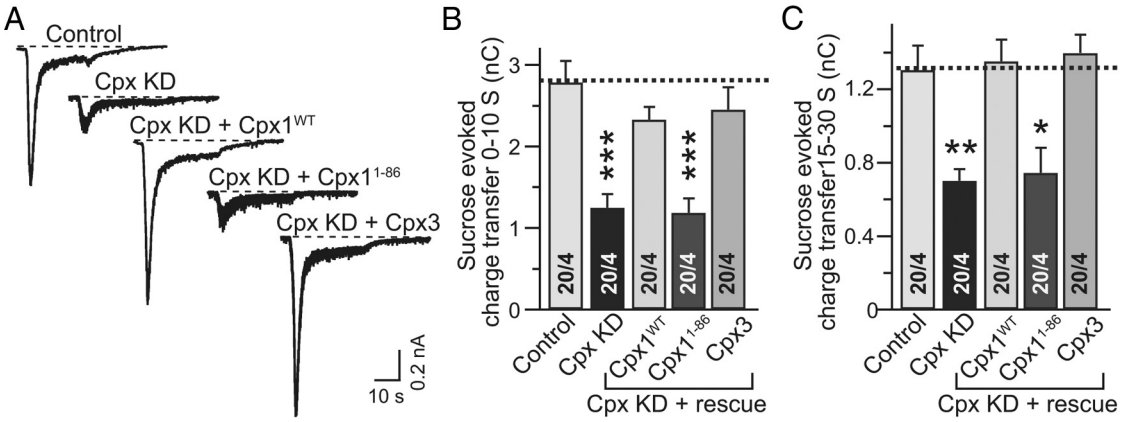

Figure 4. Analysis of the C-terminal complexin-1 sequence and of complexin-3 in priming synaptic exocytosis. ***A–C***, Sample traces of sucrose-evoked EPSCs (***A***) and summary graphs of the charge transfer induced by hypertonic sucrose during the initial 10 s phase of release (***B***) and during the maintenance phase (***C***). Release was triggered by a 30 s application of 0.5 M sucrose. Data in *B* and *C* are means ± SEMs; number of cells/independent cultures analyzed are depicted in the bars. Statistical significance was examined by Student's *t* test comparing complexin KD with control neurons (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

toxin, identified using the pClamp template program, and individually proofread (event threshold of 5 pA). Synaptic responses were triggered by a 1 ms current pulse (900 μA) through a local extracellular electrode (FHC) and recorded in whole-cell voltage-clamp mode using a Multiclamp 700A amplifier (Molecular Devices). Data were digitized at 10 kHz with a 2 kHz low-pass filter and analyzed using Clampfit 9.02 (Molecular Devices) or Igor 5.01 (Wavemetrics).

Statistical analysis. All data shown are means ± SEMs. Statistical significance was determined by Student's *t* test comparing various conditions to controls examined in the same experiments, using the number of cells as the statistical *n* with each experiment performed in at least three independent cultures, and with similar numbers of cells recorded for each condition and in each batch of culture (typically five independent cells per culture and condition). All data acquisition and analyses were performed with anonymized samples, such that the experimenter was unaware of the type of manipulation being analyzed in a sample.

Results

Complexin C-terminal sequence is required for clamping spontaneous exocytosis and for priming of vesicles but not for activation of Ca^{2+} triggering of exocytosis

We generated complexin-deficient neurons using shRNA-dependent KD of complexin-1 and complexin-2 and used rescue experiments to test the function of various complexin proteins (Maximov et al., 2009; Yang et al., 2010). These KD experiments only targeted complexin-1 and complexin-2 because complexin-3 and complexin-4 are expressed at only very low levels in the cultured cortical neurons used here. In the experiments for the present paper, we examined a series of complexin-1 mutants that were introduced into the KD neurons via the same lentivirus used for the KD and that were designed to test the functional role of the complexin-1 C-terminal sequence that accounts for nearly half its size. The various complexin mutants were robustly expressed in the cultured neurons as examined by immunoblotting (Fig. 1*B*). Immunocytochemistry showed that complexin is normally present throughout the neuron and is not a synapse-specific protein, consistent with its high expression levels and soluble nature (Fig. 1*C*; McMahon et al., 1995). As expected, the complexin KD resulted in a loss of complexin immunofluorescence signal, which was restored by expression of wild-type complexin (Fig. 1*C*).

In the first set of experiments, we compared the relative rescue efficiencies of wild-type complexin-1 (Cpx^{WT}) and of C-terminally truncated complexin-1 ($Cpx1^{1–86}$) that lacks almost the entire C-terminal complexin-1 sequence (Fig. 1*A*). Measurements of spontaneous mini exocytosis, quantified as the frequency of mEPSCs in the presence of 1 μM tetrodotoxin, revealed that C-terminally truncated complexin-1 was unable to reverse the increase in mEPSC frequency induced by the complexin KD, whereas wild-type complexin-1 fully reversed this increase (Fig. 2*A*,*B*). No effect on mEPSC amplitude was observed by any condition.

C-terminally truncated complexin-1 did, however, rescue the decrease in action-potential-evoked exocytosis in complexin-deficient neurons, demonstrating that Ca^{2+} triggering by synaptotagmin-1—which is impaired in complexin-deficient synapses (Maximov et al., 2009)—was normal (Fig. 3*A*,*B*).

Next, we measured priming by determining the size of the readily-releasable pool (RRP) of vesicles as the charge transfer mediated by synaptic responses that are induced by an application of hypertonic sucrose (Rosenmund and Stevens, 1996). Surprisingly, C-terminally truncated complexin-1 was unable to rescue the decrease in vesicle priming in complexin-deficient neurons (Fig. 4*A–C*). This result was unexpected because Ca^{2+} triggering was normal, suggesting that the Ca^{2+} triggering and priming functions of complexin-1 are mechanistically distinct. Thus, the complexin-1 C-terminal sequence is essential for its clamping and priming functions but is not required for its Ca^{2+}-triggering function. The C-terminal truncation mutant of complexin is the first mutant that dissociates its priming and Ca^{2+} functions.

Complexin-3 mediates vesicle priming and Ca^{2+} triggering of exocytosis but not clamping of spontaneous exocytosis

Complexin-1 and complexin-3 exhibit significant homology throughout their sequences except for their C-terminal region in which complexin-3, similar to complexin-4 but different from complexin-1 and complexin-2, contains an isoprenylation sequence (Fig. 1*A*; McMahon et al., 1995; Reim et al., 2005). Given this difference, we examined whether complexin-3 could functionally substitute for complexin-1 in complexin-deficient neurons. Previous studies showed that complexin-3 rescues Ca^{2+}-triggered release in complexin-deficient neurons but did not address the priming and clamping functions of complexin (Reim et al., 2005). Thus, in view of the essential role of the complexin-1 C terminus in vesicle clamping and priming, we

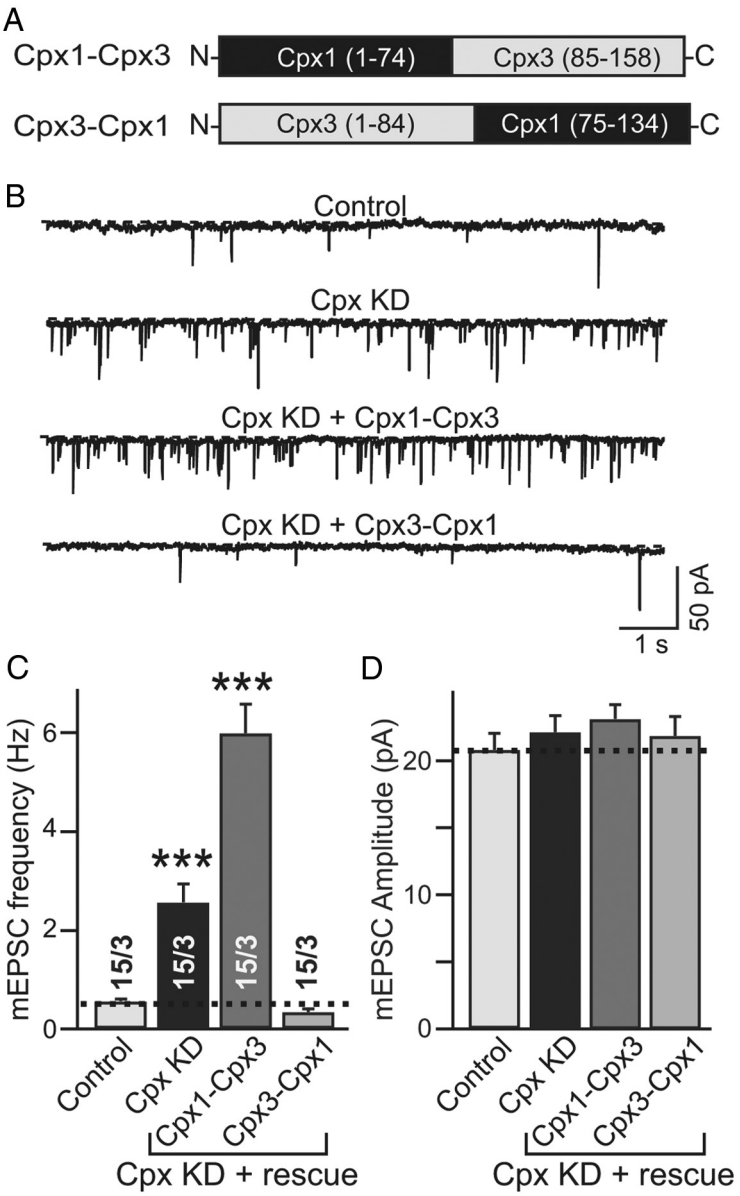

Figure 5. Localization of the difference in clamping functions between complexin-1 and complexin-3 to the C-terminal complexin sequence. ***A***, Schematic diagram of the chimeric complexin-1/3 (Cpx1–Cpx3) and complexin-3/1 proteins (Cpx3–Cpx1). For swap site sequences, see Figure 1*A*. ***B–D***, Sample traces of mEPSCs (***B***) and summary graphs of the mEPSC frequency (***C***) and amplitude (***D***) recorded in control neurons, complexin KD neurons, and complexin KD expressing the complexin-1/3 (Cpx1–Cpx3) or complexin-3/1 (Cpx3–Cpx1) proteins. Data in *C* and *D* are means ± SEMs; number of cells/independent cultures analyzed are depicted in the bars in *C*. Statistical significance was analyzed by Student's *t* test comparing all other conditions with controls (***$p < 0.001$).

systematically examined these functions in complexin-deficient neurons that express complexin-3 (Figs. 2–4).

Strikingly, complexin-3 was unable to reverse the increase in mini frequency in complexin-deficient neurons (Fig. 2*A–C*) but completely rescued their impairment in Ca^{2+} triggering (Fig. 3). Moreover, complexin-3 rescued the decrease in priming induced by the complexin KD (Fig. 4). Thus, complexin-1 and complexin-3—despite a modest degree of sequence identity (Fig. 1*A*)—perform similar functions, except that complexin-3 does not clamp spontaneous mini release.

The difference in clamping functions of complexin-1 and complexin-3 localizes to their C-terminal sequences

To determine whether complexin-3 is unable to clamp mini release because of sequence differences with complexin-1 in the accessory α-helix that was shown previously to be essential for clamping (Maximov et al., 2009; Xue et al., 2010) or in the C-terminal region that we here showed to be essential for clamping (Fig. 1), we generated chimeric complexin-1 and complexin-3 proteins in which the three N-terminal regions (the N-terminal activation domain and the two α-helices) are derived from one complexin isoform and the C-terminal sequence from the other complexin isoform (Fig. 5*A*). Rescue experiments revealed that complexin-1/3 composed of the three N-terminal complexin-1 regions and the C-terminal complexin-3 sequence aggravated the loss of clamping in complexin-deficient neurons, resulting in an even higher mini frequency (Fig. 5*B–D*). In contrast, complexin-3/1, composed of the three N-terminal complexin-3 regions and the C-terminal complexin-1 sequence, fully reversed the increase in mini frequency, i.e., was fully capable of clamping spontaneous release. Both chimeric complexin proteins fully rescued the decrease in Ca^{2+}-triggered exocytosis in complexin-deficient neurons (Fig. 6). Thus, the inability of complexin-3 to clamp mini release is based on its C-terminal sequence, possibly because the membrane anchoring of complexin-3 via its C-terminal isoprenylation blocks its clamping function by an unknown mechanism.

Point mutations in the C-terminal complexin-1 sequence inhibit its clamping function

To further understand how the C terminus of complexin contributes to the regulation of spontaneous mini release, we searched for point mutations within the C terminus that can alter this function. The complexin C terminus is predicted to be primarily unstructured, except for a possible amphipathic α-helix that is conserved among human, mouse, and *C. elegans* complexins (Fig. 1*A*; Seiler et al., 2009). Based on previously published mutants that alter complexin–liposome binding (Seiler et al., 2009), we generated four point mutations in or near the putative amphipathic α-helix: leucine-117 is replaced by either a bulky hydrophobic tryptophan (Cpx^{L117W}) or a charged lysine (Cpx^{L117K}), valine-120 is replaced by a proline (Cpx^{V120P}) to disrupt the α-helix, and isoleucine-101 is replaced with a tryptophan (Cpx^{I101W}; Fig. 1*A*).

mEPSC recordings showed that the Cpx^{I101W} and Cpx^{L117W} mutations did not impair rescue of the increase in spontaneous mini release in Cpx KD neurons, whereas the Cpx^{L117K} and Cpx^{V120P} mutations blocked this rescue (Fig. 7*A–C*). However, all four mutants fully rescued EPSC amplitude (Fig. 8).

Function of the C-terminal complexin sequence in release induced by 10 Hz stimulus trains

To test the role of the complexin-1 C-terminal sequence in release induced by stimulus trains and to clarify whether the clamping function of complexin-1 that requires its C-terminal sequence is also involved in limiting delayed release (a form of asynchronous release that occurs after the last action potential in a stimulus train, when synchronous release has ended; Maximov and Südhof, 2005), we analyzed NMDA-receptor-mediated EPSCs under blockage of AMPA receptors. AMPA-receptor-mediated release induced by stimulus trains cannot be easily analyzed in cultured neurons because of the strong network activity in these cultures (Fig. 6*A*), which can be blocked when NMDA-receptor-mediated EPSCs are examined in postsynaptically depolarized neurons (Fig. 9*A*). To ensure that measurements of AMPA- and NMDA-receptor-mediated release produces the same overall conclusions, we first examined the effect of the Cpx KD with rescue by truncated complexin-1 ($Cpx1^{1-86}$) and two complexin-1 point mutants ($Cpx1^{L117W}$ and $Cpx1^{L117K}$) on release induced by isolated action potentials (Fig. 9*A*,*B*). We found that the complexin KD caused the same decrease in the EPSC when measured via NMDA- or AMPA-receptor-mediated responses and that this decrease was similarly rescued by the various complexin-1 mutants (Fig. 9*B*). Thus, the NMDA receptor response precisely correlates with the AMPA receptor response under the present conditions.

Next, we examined the function of the complexin-1 C-terminal sequences on release induced by a 10 Hz stimulus train (Fig. 9*C*,*D*). The synaptic response to the first action potential and to most of the following action potentials is synchronous, although an asynchronous component increasingly contributes to the responses during the train; in addition, all release after the end of the train (the so-called delayed release) is asynchronous (Maximov and Südhof, 2005). Asynchronous and synchronous release are derived from the same RRP and therefore dependent on the size of RRP (Hagler and Goda, 2001; Otsu et al., 2004).

All three C-terminal mutants tested (Cpx^{1-86}, Cpx^{L117W}, and Cpx^{L117K}) rescued the decreased release during the entire train in complexin-deficient neurons (Fig. 9*C*,*D*). A similar pattern was observed for delayed release, whereas the ratio of delayed-to-total release was modestly but significantly increased by the complexin KD and after Cpx^{1-86} rescue (Fig. 9*C*,*D*). It is thought that both total release and delayed release depend on the RRP size, that the total release is primarily dependent on synchronous Ca^{2+}-triggered release, and that delayed release is primarily dependent on asynchronous Ca^{2+}-triggered release. Thus, the increased ratio of delayed-to-total release in both complexin KD and Cpx^{1-86} rescue neurons is consistent with the notion that the complexin C-terminal sequences clamp asynchronous Ca^{2+}-triggered release but prime overall release, leading to a relatively higher ratio of delayed-to-total release in the stimulus train experiments (Fig. 9*D*).

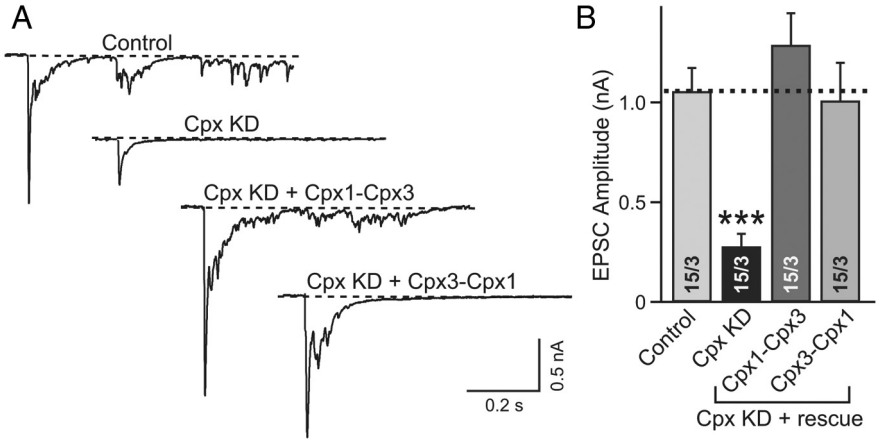

Figure 6. Chimeric complexin-1 and complexin-3 proteins fully rescue Ca^{2+}-triggered exocytosis in complexin-deficient neurons. ***A***, ***B***, Sample traces of action-potential-evoked EPSCs (***A***) and summary graphs of EPSC amplitudes (***B***). Note the abundant network activity of EPSCs in cultured neurons when AMPA-mediated EPSCs are analyzed, which prevents reliable estimation of any parameter except for the initial EPSC amplitude. Data in *B* are means ± SEMs; number of cells/independent cultures analyzed are depicted in the bars. Statistical significance was analyzed by Student's *t* test comparing all other conditions with controls (***$p < 0.001$).

Discussion

In the present study, we have explored the function of the C-terminal sequences of complexins, relatively large sequences that account for nearly half of the complexin protein but nevertheless have remained functionally incompletely characterized. Because complexin-1 and complexin-2, and complexin-3 and complexin-4 form homologous pairs, we tested the effect of deleting or mutating the C-terminal sequences of complexin-1 and complexin-3, asking among others whether complexin-1 and complexin-3 perform similar functions in synaptic exocytosis and whether the functional differences between these complexins localize to their C-terminal regions. We made the following principal observations.

(1) Deletion of the complexin-1 C-terminal sequence revealed that it is essential for both its priming and clamping function but not for its synaptotagmin-activating function, thereby dissociating the two "positive" functions (synaptotagmin-activating and priming) of complexin.

(2) Complexin-1 and complexin-3 have similar priming and Ca^{2+}-triggering functions but dramatically differ from each other in that only complexin-1 but not complexin-3 mediates synaptic vesicle clamping of spontaneous mini release. This result is consistent with studies performed in *Drosophila* (Cho et al., 2010).

(3) Swapping the C-terminal sequences of complexin-1 and complexin-3 showed that the complexin-3 C-terminal sequence inactivates the complexin-1 clamping function, whereas the complexin-1 C-terminal sequence enables synaptic clamping by complexin-3. Thus, the functional differences between these two isoforms are entirely based on the differences between their C-terminal sequences.

(4) Two point mutations in the predicted amphipathic C-terminal α-helix of complexin-1 that binds to phospholipids (Seiler et al., 2009) also block the clamping function of complexin-1, suggesting that phospholipid binding by complexin is important for its clamping function.

Viewed together, our data demonstrate that the C-terminal complexin-1 sequence critically contributes to its overall function and show that complexin-3 performs similar functions as complexin-1, except that, at least in our system, complexin-3

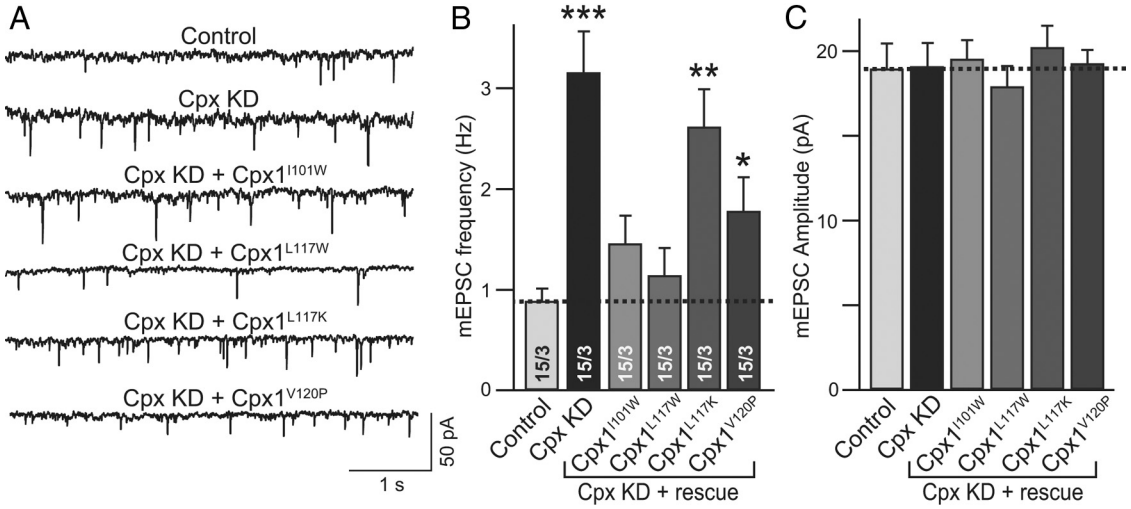

Figure 7. Complexin-1 point mutations implicated in lipid binding impair the clamping function of complexin-1. ***A–C***, Sample traces of mEPSCs (***A***) and summary graphs of the mEPSC frequency (***B***) and amplitude (***C***) recorded in control neurons and in complexin KD neurons without or with rescue with the indicated complexin proteins (I101W substitution, Cpx1^{I101W}; L117W substitution, Cpx1^{L117W}; L117K substitution, Cpx1^{L117K}; and V120P substitution, Cpx1^{V120P}) mutations. All data are means ± SEMs; number of cells/independent cultures analyzed are depicted in the bars. Statistical significance was analyzed by Student's *t* test comparing all other conditions with controls (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

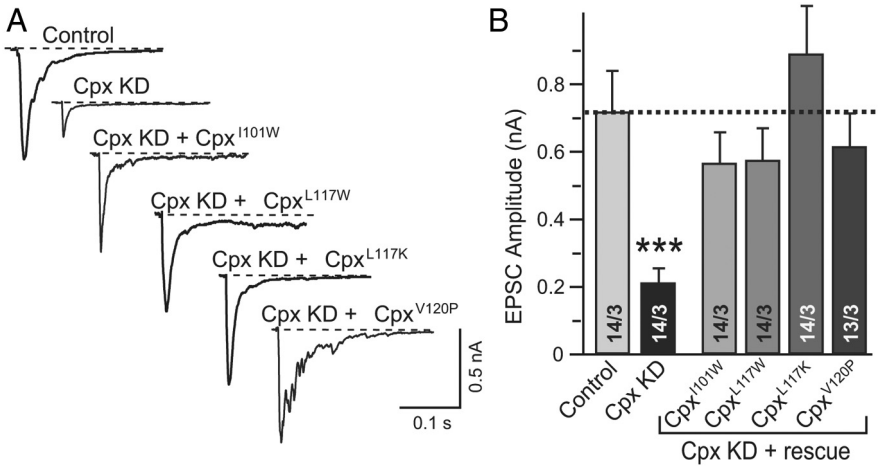

Figure 8. Complexin-1 point mutations implicated in lipid binding retains its activity in Ca^{2+}-triggered exocytosis. ***A***, ***B***, Sample traces of action-potential-evoked EPSCs (***A***) and summary graphs of EPSC amplitudes (***B***) recorded in control neurons and in complexin KD neurons without or with rescue with the indicated complexin proteins (Cpx1^{I101W}, Cpx1^{L117W}, Cpx1^{L117K}, and Cpx1^{V120P}) mutations. Data in *B* are means ± SEMs; number of cells/independent cultures analyzed are depicted in the bars. Statistical significance was analyzed by Student's *t* test comparing all other conditions with controls (***$p < 0.001$).

does not clamp fusion and that this functional difference localizes to the C-terminal sequence of complexin-1 and complexin-3.

It is surprising that a small molecule such as complexin-1 with only 134 residues mediates three independent functions during synaptic exocytosis (activation of synaptotagmin function, clamping, and priming) and that these functions within the already small complexin molecule selectively depend on distinct short modular sequences (Table 1). The overall design of complexin appears to involve the linkage of different peripheral functional modules that mediate activation of Ca^{2+} triggering, priming, and clamping to the same central functional module, namely the SNARE-binding α-helix that is essential for all of these functions (Chen et al., 2002; Tang et al., 2006; Maximov et al., 2009).

Our results extend some of the previous studies but are inconsistent with others. Experiments with liposome fusion suggested that the C-terminal complexin-1 but not complexin-3 sequence facilitates membrane fusion and that this function can be transferred from complexin-1 to complexin-3 in complexin-3/1 chimera (Seiler et al., 2009). In our more physiological assay system that monitors synaptic exocytosis in cultured primary neurons, however, complexin-3 activates fusion as well as complexin-1, confirming previous results (Reim et al., 2005). Thus, the liposome results may be particular to liposomes as a reduced system. Moreover, we find that complexin-3 in mouse neurons does not clamp mini release, whereas in *Drosophila* the same complexin isoform was found to clamp release (Cho et al., 2010), possibly as a result of species differences. Furthermore, our conclusion that the C terminus of complexin-1 is essential for its clamping function agrees well with studies in *C. elegans* that arrived at the same conclusion based on a genetic approach (Martin et al., 2011). Finally, a long-term unresolved issue about complexin has been the question why KD of complexins in cultured neurons produces an unclamping of mini release (Maximov et al., 2009; Yang et al., 2010), whereas knock-out of complexins in autapses or in slices does not (Reim et al., 2001; Xue et al., 2007; Strenzke et al., 2009), although more recent studies have also discovered a clamping function for complexins in autapses (Xue et al., 2010). This issue remains unresolved, because it is still unclear why the "unclamping " phenotype is so much less pronounced in autapses from knock-out neurons than in synapses between cultured neurons after KD of complexins. However, there appears to be general agreement that the activating functions of complexin are more important than its clamping functions, and it is possible that the clamping function is more dependent on the experimental context than the activating functions of complexin.

We do not know the precise mechanisms by which the C-terminal complexin sequences contribute to vesicle priming

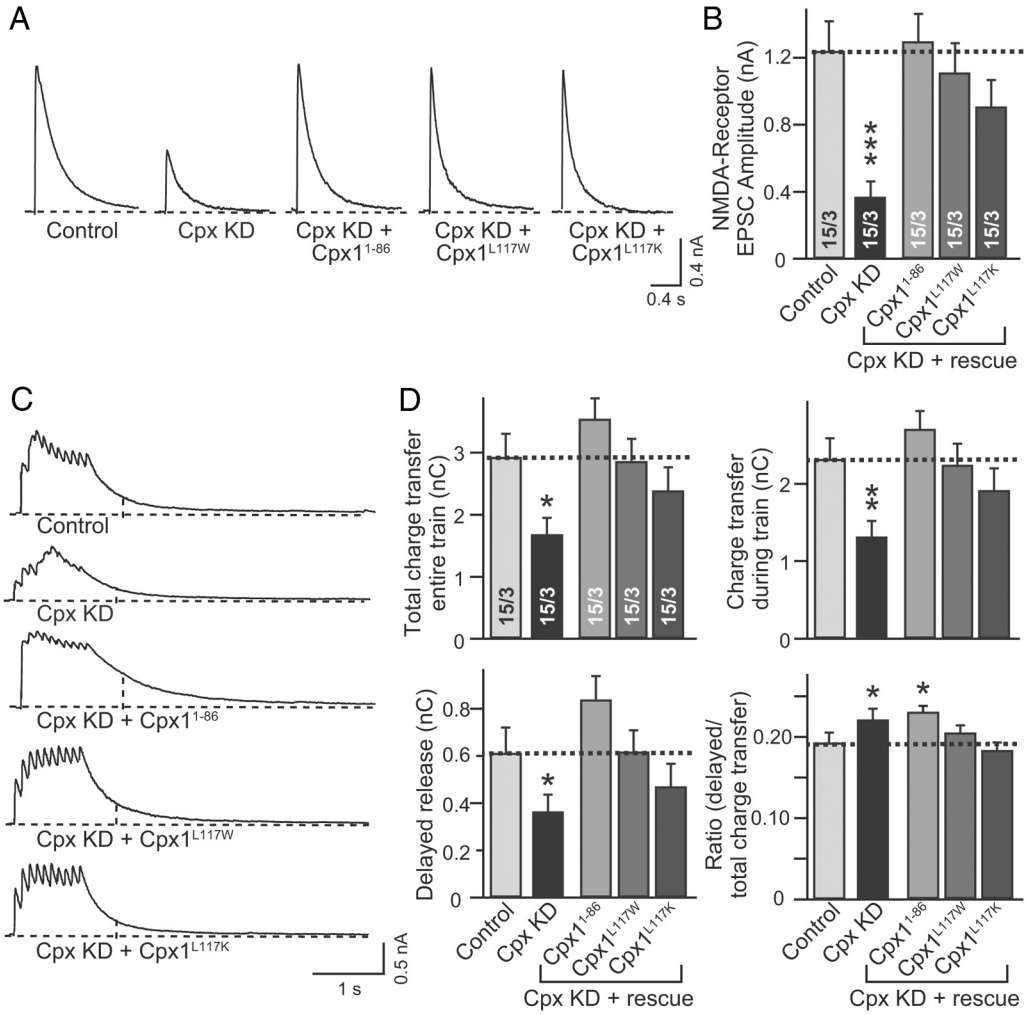

Figure 9. Effects of the complexin-1 C-terminal truncation mutation and of complexin-1 point mutations on exocytosis triggered by stimulus trains and analyzed as NMDA-receptor-mediated EPSCs to avoid network activity. ***A***, ***B***, Sample traces of NMDA-receptor-dependent EPSCs (***A***) and summary graphs of EPSC amplitudes (***B***) evoked by isolated action potentials in control neurons, complexin KD neurons without and with rescue with the complexin C-terminal truncation (Cpx $^{1-86}$), complexin L117W (Cpx ^{L117W}), L117K (Cpx ^{L117K}) mutations. All analyses in this figure were performed in the presence of the AMPA receptor blocker CNQX (20 μM). ***C***, Sample traces of NMDA-receptor-mediated EPSCs evoked by action-potential trains (10 Hz for 1 s). Vertical dashed line indicates the cutoff time for calculation of delayed release. ***D***, Summary graphs of various parameters of synaptic transmission induced by a 10 Hz, 1 s stimulus train, quantified as the synaptic charge transfer. Four parameters were analyzed: total synaptic charge transfer during and after the train (top left) or only during the train (top right), mean synaptic charge transfer during delayed release starting 500 ms after the last action potential (bottom left), and mean ratio of delayed release to the total charge transfer (bottom right). All data in *B* and *D* are means ± SEMs; number of cells/independent cultures analyzed are depicted in the bars (the numbers in the top left diagram in *D* apply to all diagrams because the same experiments were analyzed). Statistical significance was assessed by Student's *t* test comparing all other conditions with controls (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Table 1. Rescue phenotypes of cortical synapses with complexin-1/2 double knockdown expressing the indicated complexin forms

Complexin used for rescue	Vesicle priming	Vesicle clamping	Fast Ca^{2+} triggering
No rescue	↓	↓	↓
Complexin-1	↑	↑	↑
Complexin-3	↑	↓	↑
Complexin-1 $^{1-86}$	↓	↓	↑
Complexin-1/3 hybrid	n.d.	↓	↑
Complexin-3/1 hybrid	n.d.	↑	↑
Complexin-1 ^{I101W}	n.d.	↑	↑
Complexin-1 ^{L117W}	n.d.	↑	↑
Complexin-1 ^{L117K}	n.d.	↓	↑
Complexin-1 ^{V120P}	n.d.	↓	↑

Summary of the complexin KD and rescue experiment results. Complexin-1/2 KD results in reduced vesicle priming and clamping and decreased Ca^{2+} triggering of fast release. The efficacy of various complexin proteins to rescue the three features of the KD phenotype is indicated (↑ , rescue; ↓ , no rescue; n.d., not determined).

and clamping. A plausible hypothesis, however, is based on the finding that the amphipathic α-helix in the C-terminal complexin-1 sequence binds to lipid bilayers (Malsam et al., 2009; Seiler et al., 2009). It is tempting to speculate that such binding blocks fusion and that mutations in the α-helix reverse this block. However, this hypothesis does not explain the role of the complexin-1 C-terminal sequence in priming and raise additional questions, such as why the accessory α-helix is additionally required for clamping (Maximov et al., 2009). Future studies will have to address these important issues.

References

Bracher A, Kadlec J, Betz H, Weissenhorn W (2002) X-ray structure of a neuronal complexin-SNARE complex from squid. J Biol Chem 277:26517–26523.

Cai H, Reim K, Varoqueaux F, Tapechum S, Hill K, Sørensen JB, Brose N, Chow RH (2008) Complexin II plays a positive role in Ca^{2+}-triggered

exocytosis by facilitating vesicle priming. Proc Natl Acad Sci U S A 105:19538–19543.

Chen X, Tomchick DR, Kovrigin E, Ara ç D, Machius M, Südhof TC, Rizo J (2002) Three-dimensional structure of the complexin/SNARE complex. Neuron 33:397–409.

Cho RW, Song Y, Littleton JT (2010) Comparative analysis of *Drosophila* and mammalian complexins as fusion clamps and facilitators of neurotransmitter release. Mol Cell Neurosci 45:389–397.

Fernández-Chacón R, Königstorfer A, Gerber SH, García J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C, Südhof TC (2001) Synaptotagmin I functions as a calcium regulator of release probability. Nature 410:41–49.

Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Südhof TC (1994) Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. Cell 79:717–727.

Giraudo CG, Eng WS, Melia TJ, Rothman JE (2006) A clamping mechanism involved in SNARE-dependent exocytosis. Science 313:676–680.

Giraudo CG, Garcia-Diaz A, Eng WS, Yamamoto A, Melia TJ, Rothman JE (2008) Distinct domains of complexins bind SNARE complexes and clamp fusion in vitro. J Biol Chem 283:21211–21219.

Giraudo CG, Garcia-Diaz A, Eng WS, Chen Y, Hendrickson WA, Melia TJ, Rothman JE (2009) Alternative zippering as an on-off switch for SNARE-mediated fusion. Science 323:512–516.

Hagler DJ Jr, Goda Y (2001) Properties of synchronous and asynchronous release during pulse train depression in cultured hippocampal neurons. J Neurophysiol 85:2324–2334.

Hobson RJ, Liu Q, Watanabe S, Jorgensen EM (2011) Complexin maintains vesicles in the primed state in *C. elegans*. Curr Biol 21:106–113.

Huntwork S, Littleton JT (2007) A complexin fusion clamp regulates spontaneous neurotransmitter release and synaptic growth. Nat Neurosci 10:1235–1237.

Malsam J, Seiler F, Schollmeier Y, Rusu P, Krause JM, Söllner TH (2009) The carboxy-terminal domain of complexin I stimulates liposome fusion. Proc Natl Acad Sci U S A 106:2001–2006.

Martin JA, Hu Z, Fenz KM, Fernandez J, Dittman JS (2011) Complexin has opposite effects on two modes of synaptic vesicle fusion. Curr Biol 21:97–105.

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

Maximov A, Tang J, Yang X, Pang ZP, Südhof TC (2009) Complexin controls the force transfer from SNARE complexes to membranes in fusion. Science 323:516–521.

McMahon HT, Missler M, Li C, Südhof TC (1995) Complexins: cytosolic proteins that regulate SNAP receptor function. Cell 83:111–119.

Otsu Y, Shahrezaei V, Li B, Raymond LA, Delaney KR, Murphy TH (2004) Competition between phasic and asynchronous release for recovered synaptic vesicles at developing hippocampal autaptic synapses. J Neurosci 24:420–433.

Pang ZP, Xu W, Cao P, Südhof TC (2010) Calmodulin suppresses synaptotagmin-2 transcription in cortical neurons. J Biol Chem 285:33930–33939.

Reim K, Mansour M, Varoqueaux F, McMahon HT, Südhof TC, Brose N, Rosenmund C (2001) Complexins regulate a late step in Ca^{2+}-dependent neurotransmitter release. Cell 104:71–81.

Reim K, Wegmeyer H, Brandstätter JH, Xue M, Rosenmund C, Dresbach T, Hofmann K, Brose N (2005) Structurally and functionally unique complexins at retinal ribbon synapses. J Cell Biol 169:669–680.

Rosenmund C, Stevens CF (1996) Definition of the readily releasable pool of vesicles at hippocampal synapses. Neuron 16:1197–1207.

Schaub JR, Lu X, Doneske B, Shin YK, McNew JA (2006) Hemifusion arrest by complexin is relieved by Ca^{2+}-synaptotagmin I. Nat Struct Mol Biol 13:748–750.

Seiler F, Malsam J, Krause JM, Söllner TH (2009) A role of complexin-lipid interactions in membrane fusion. FEBS Lett 583:2343–2348.

Strenzke N, Chanda S, Kopp-Scheinpflug C, Khimich D, Reim K, Bulankina AV, Neef A, Wolf F, Brose N, Xu-Friedman MA, Moser T (2009) Complexin-I is required for high-fidelity transmission at the endbulb of Held auditory synapse. J Neurosci 29:7991–8004.

Südhof TC, Rothman JE (2009) Membrane fusion: grappling with SNARE and SM proteins. Science 323:474–477.

Tang J, Maximov A, Shin OH, Dai H, Rizo J, Südhof TC (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell 126:1175–1187.

Tokumaru H, Shimizu-Okabe C, Abe T (2008) Direct interaction of SNARE complex binding protein synaphin/complexin with calcium sensor synaptotagmin 1. Brain Cell Biol 36:173–189.

Xue M, Reim K, Chen X, Chao HT, Deng H, Rizo J, Brose N, Rosenmund C (2007) Distinct domains of complexin I differentially regulate neurotransmitter release. Nat Struct Mol Biol 14:949–958.

Xue M, Lin YQ, Pan H, Reim K, Deng H, Bellen HJ, Rosenmund C (2009) Tilting the balance between facilitatory and inhibitory functions of mammalian and *Drosophila* Complexins orchestrates synaptic vesicle exocytosis. Neuron 64:367–380.

Xue M, Craig TK, Xu J, Chao HT, Rizo J, Rosenmund C (2010) Binding of the complexin N terminus to the SNARE complex potentiates synaptic-vesicle fusogenicity. Nat Struct Mol Biol 17:568–575.

Yang X, Kaeser-Woo YJ, Pang ZP, Xu W, Südhof TC (2010) Complexin clamps asynchronous release by blocking a secondary Ca^{2+} sensor via its accessory alpha helix. Neuron 68:907–920.

Yoon TY, Lu X, Diao J, Lee SM, Ha T, Shin YK (2008) Complexin and Ca^{2+} stimulate SNARE-mediated membrane fusion. Nat Struct Mol Biol 15:707–713.