Deconstructing complexin function in activating and clamping Ca^{2+}-triggered exocytosis by comparing knockout and knockdown phenotypes

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Complexin, a presynaptic protein that avidly binds to assembled SNARE complexes, is widely acknowledged to activate Ca^{2+}-triggered exocytosis. In addition, studies of invertebrate complexin mutants and of mouse neurons with a double knockdown (DKD) of complexin-1 and -2 suggested that complexin maintains the readily releasable pool (RRP) of vesicles and clamps spontaneous exocytosis. In contrast, studies of mouse neurons with a double knockout (DKO) of complexin-1 and -2, largely carried out in hippocampal autapses, did not detect changes in the RRP size or in spontaneous exocytosis. To clarify complexin function, we here directly compared in two different preparations, cultured cortical and olfactory bulb neurons, the phenotypes of complexin DKD and DKO neurons. We find that complexin-deficient DKD and DKO neurons invariably exhibit a ∼50% decrease in vesicle priming. Moreover, the DKD consistently increased spontaneous exocytosis, but the DKO did so in cortical but not olfactory bulb neurons. Furthermore, the complexin DKD but not the complexin DKO caused a compensatory increase in complexin-3 and -4 mRNA levels; overexpression of complexin-3 but not complexin-1 increased spontaneous exocytosis. Complexin-3 but not complexin-1 contains a C-terminal lipid anchor attaching it to the plasma membrane; addition of a similar lipid anchor to complexin-1 converted complexin-1 from a clamp into an activator of spontaneous exocytosis. Viewed together, our data suggest that complexin generally functions in priming and Ca^{2+} triggering of exocytosis, and additionally contributes to the control of spontaneous exocytosis dependent on the developmental history of a neuron and on the subcellular localization of the complexin.

synaptic transmission | neurotransmitter release | synaptotagmin | SNARE protein | membrane fusion

Complexin is a small, evolutionarily conserved protein that binds to SNARE complexes and has been variably associated with clamping spontaneous synaptic vesicle exocytosis ("mini release"), priming vesicles for exocytosis, and assisting synaptotagmin in Ca^{2+} triggering of exocytosis (1, 2). Four complexins are expressed in brain. The highly abundant complexin-1 (Cpx1) and -2 (Cpx2) are soluble proteins (3), whereas the low-abundance complexin-3 (Cpx3) and -4 (Cpx4) are attached to plasma membranes by a C-terminal lipid anchor (4). Complexins are conserved in invertebrates, which usually also express soluble and membrane-anchored forms (5, 6). In human patients, significant evidence links complexin expression to schizophrenia, although it is unclear whether the observed changes are cause or consequence of the disorder (7–10).

All complexins are composed of four domains that are packed into a short ∼130-residue sequence. These domains are composed of unstructured N-terminal and C-terminal regions that flank adjacent accessory and central α-helices (11). In Cpx1 and Cpx2, the N-terminal region is required for Ca^{2+} triggering of release, the accessory α-helix is essential for clamping release, the central α-helix binds to assembling SNARE complexes, and the C-terminal region is essential for both priming and clamping vesicles (5, 6, 11–18). Loss-of-function experiments combined with rescues demonstrated that the three principal functions of complexins (in priming, clamping, and Ca^{2+} triggering of exocytosis) are independent of each other because some mutations selectively impair one or two of the three functions, but leave the other function(s) intact (14, 15, 17). However, all complexin functions require its central α-helix that binds to SNARE complexes, suggesting that complexin always acts in association with SNARE complexes (14).

It is generally agreed that complexins activate Ca^{2+}-triggered exocytosis, but considerable uncertainty exists about the relative importance of the other two functions of complexin, i.e., their role in priming and clamping exocytosis. Most of this uncertainty is based on differences in approach. For example, in vitro fusion assays initially only described a clamping function of complexin, thus emphasizing this role (19–22). Analysis of mouse complexin double-knockout (DKO) neurons lacking Cpx1 and Cpx2, conversely, initially demonstrated only a Ca^{2+}-triggering function of complexin, thus suggesting that mammalian complexins primarily function in this role (4, 13). Later experiments with liposomes, however, also revealed an activating function for complexin (23–26). Conversely, subsequent experiments suggested that the energy barrier to fusion may be increased in complexin DKO neurons, but did not detect an alteration in the total size of the readily releasable pool (RRP) of vesicles (16). In contrast to the DKO studies, studies with neurons overexpressing dominant-negative Cpx1 (12) or with double-knockdown (DKD) neurons containing dramatically reduced Cpx1 and Cpx2 levels identified phenotypes in all three

Significance

Complexin, a presynaptic protein that avidly binds to assembled SNARE complexes, is known to activate Ca^{2+}-triggered exocytosis of synaptic vesicles. However, the function of complexin in priming the readily releasable pool of vesicles and in clamping spontaneous exocytosis remained debatable because knockout and knockdown approaches produced different results. Here, in a direct comparison of these approaches, we find that complexin-deficient neurons invariably exhibited a decrease in vesicle priming, but that their spontaneous release phenotype depended on the developmental history and type of neuron. Viewed together, our data suggest that complexin generally functions in priming and Ca^{2+} triggering of exocytosis, and additionally, dependent on the developmental context, contributes to the control of spontaneous exocytosis.

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presumed functional domains of complexin—clamping, priming, and Ca^{2+} triggering of exocytosis (14, 15, 17). Furthermore, studies of genetic complexin mutants in *Drosophila* and *Caenorhabditis elegans* also revealed phenotypes in clamping, priming, and Ca^{2+} triggering (5, 6, 18, 27–30).

Viewed together, the studies in invertebrates and DKD neurons broadly agreed in suggesting complexin functions in the clamping, priming, and Ca^{2+} activation of exocytosis, whereas the studies in DKO neurons indicated a more restricted complexin function in Ca^{2+} activation of exocytosis. Because a genetic DKO is likely technically more reliable than an shRNA-mediated DKD, the discrepancies between the DKO and DKD results in mouse neurons give rise to concern. As a further complicating factor, the DKO studies were largely carried out in autapses in which a hippocampal neuron is grown in isolation so that it forms synapses on itself, whereas the DKD experiments were performed on synapses formed between neurons cultured at high density.

The confusion about the functions of complexin has hindered our understanding of the mechanism of exocytosis. Increasing numbers of studies indicate that complexin performs a fundamental general function in Ca^{2+}-stimulated exocytosis that is not limited to the synapse. For example, complexin is involved in chromaffin granule exocytosis, which resembles the exocytosis of large dense-core vesicles (31), and is essential for Ca^{2+}-induced exocytosis of IGF1-containing secretory vesicles in mitral neurons that depends on a different synaptotagmin isoform (Syt10) than synaptic and neuroendocrine exocytosis (Syt1, Syt2, Syt7, and Syt9) (32). A major cause for the uncertainty of complexin function at least in mammalian neurons is that the phenotypes of the DKO and DKD neurons have not been directly compared in the same electrophysiological preparation. Therefore, we have in the present study systematically examined the effects of the separate or combined DKO and DKD manipulations on synaptic transmission in two different types of cultured neurons, cortical neurons and olfactory bulb neurons.

Results

Complexins Are Essential for Vesicle Priming into the RRP. We cultured cortical and olfactory bulb neurons from newborn littermate Cpx2 KO mice, which are viable and fertile and exhibit no major phenotype, and from Cpx1/2 DKO mice, which die at birth and exhibit a severe impairment in Ca^{2+}-triggered neurotransmitter release (4). Neurons were cultured at high density and form extensive synaptic connections (33), were infected with a control lentivirus or with the Cpx1/2 DKD lentivirus (14), and were analyzed for action potential- as well as hypertonic sucrose-evoked excitatory postsynaptic currents (EPSCs) (Fig. 1 *A–D*). Action potentials evoke neurotransmitter release by inducing Ca^{2+}-triggered exocytosis, whereas hypertonic sucrose stimulates the Ca^{2+}-independent exocytosis of vesicles in the RRP, and thus allows measurement of the RRP size (34). We performed these experiments in parallel in both cortical and olfactory bulb neurons to ensure the generality of all observations.

In both cortical and olfactory bulb neurons, we observed a large (approximately fivefold) decrease in the amplitude of action potential-evoked EPSCs in DKD, DKO, and DKD/DKO neurons compared with control Cpx2 KO neurons (Fig. 1 *A* and *C*). The DKD/DKO combination produced a marginally but significantly stronger EPSC reduction than either manipulation alone. We also observed a more than twofold reduction in the RRP in DKD, DKO, and DKD/DKO neurons compared with Cpx2 KO control neurons, with again a significantly larger RRP reduction produced by the DKD/DKO combination (Fig. 1 *B* and *D*). No major change in the kinetics of sucrose-induced EPSCs was observed. The decrease in the RRP induced by the Cpx1/2 DKD and DKO is consistent with previous results suggesting that complexins are essential for synaptic vesicle priming (15, 17, 28, 31). All DKO phenotypes were rescued in cortical neurons by wild-type Cpx1, demonstrating that they are produced by the complexin deletion (Fig. S1). Thus, Cpx1/2 DKD and DKO neurons consistently reveal a large and essential role for complexins in vesicle priming and in Ca^{2+} triggering of vesicle exocytosis.

Variable Complexin Function in Clamping Spontaneous Mini Release. We next measured spontaneous miniature excitatory currents (mEPSCs) as well as the capacitance and input resistance in complexin-deficient cortical (Fig. 2 *A* and *B*) and olfactory bulb neurons (Fig. 2 *C* and *D*).

We found that, in cortical neurons, the DKD increased the mEPSC frequency almost fivefold when applied to Cpx2 KO neurons, whereas the DKO increased the mEPSC frequency

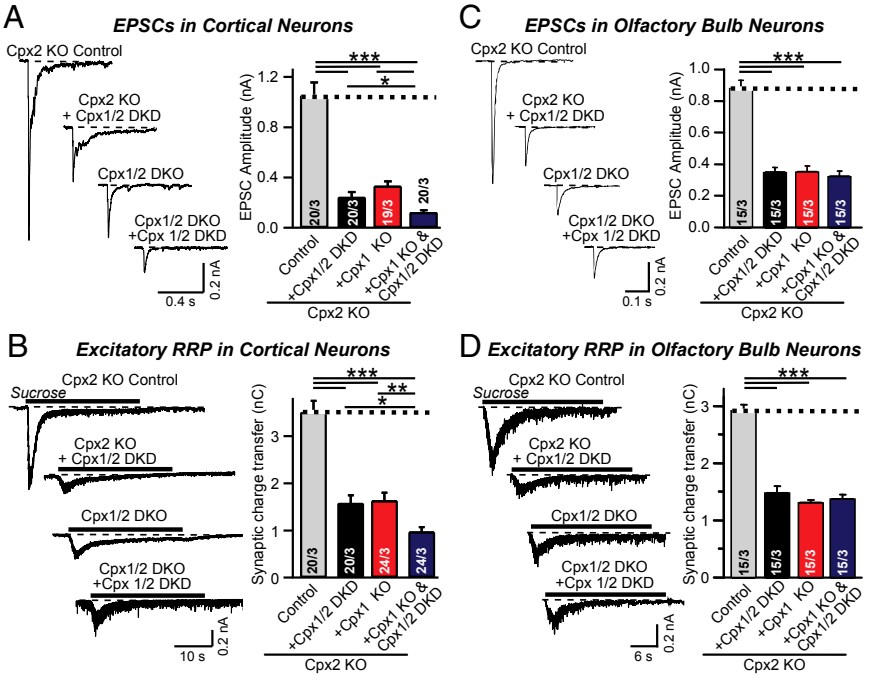

Fig. 1. Complexin activates neurotransmitter release evoked by action potentials or hypertonic sucrose in cortical and olfactory bulb neurons. (*A*) Sample traces (*Left*) and summary graphs of the amplitude (*Right*) of action-potential evoked EPSCs monitored in cortical Cpx2 KO neurons without and with additional KO of Cpx1 (Cpx1/2 DKO), and with or without the Cpx1/2 DKD. (*B*) Sample traces (*Left*) and summary graphs of the charge transfer of EPSCs evoked by 0.5 M sucrose (*Right*; integrated over 30 s), recorded from cortical neurons as described for *A*. (*C* and *D*) Same as *A* and *B*, but measured in olfactory bulb neurons. All data are means ± SEM; numbers of neurons/independent cultures analyzed are depicted in bars. Statistical significance was analyzed by Student *t* test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

approximately twofold (Fig. 2*A*). Combining the DKD and DKO produced the same increase in mEPSC frequency as the DKD alone. None of the manipulations altered the mEPSC amplitude (Fig. 2*A*), or affected neuronal capacitance or input resistance (Fig. 2*B*). Thus, the DKD and DKO of complexins both "unclamp" the mEPSC frequency in cortical neurons, although the relative effect of the DKD is stronger, and the DKD paradoxically further unclamps mEPSCs in DKO neurons.

In olfactory bulb neurons, the DKD had the same unclamping effect on mEPSC frequency as in cortical neurons (Fig. 2*C*). Strikingly, however, the DKO decreased the mEPSC frequency in olfactory bulb neurons compared with the Cpx2 KO control. The combination of the DKD and DKO again replicated the DKD phenotype, reversing the mESPC frequency decrease produced by the DKO (Fig. 2*C*). Again, no effect on mEPSC amplitude was observed by any manipulation.

In addition, in olfactory bulb neurons both the DKD and the DKO decreased the neuronal capacitance and increased the neuronal input resistance compared with Cpx2 KO neurons (Fig. 2*D*). This result is consistent with previous observations that, in olfactory bulb neurons, complexins are essential for IGF1 exocytosis, leading to an impairment in neuronal growth in the absence of complexins (32).

The Complexin DKD but Not the DKO Alter Cpx3 and Cpx4 mRNA Levels. To explore why the DKO and DKD provide similar phenotypes in evoked release and electrical properties, but divergent phenotypes in spontaneous mini release, we asked whether compensatory changes in the expression of other complexins may occur in response to these manipulations. We performed quantitative RT-PCR measurements of Cpx1, Cpx3, and Cpx4 mRNAs in Cpx2 KO and Cpx1/2 DKO cortical neurons that were infected either with control or with the Cpx1/2 DKD lentivirus. We then plotted the measured mRNA levels both in terms of absolute mRNA levels normalized to GAPDH (Fig. 3, *Upper*), and as relative mRNA levels of the DKD condition compared with the Cpx2 KO or the DKO alone (Fig. 3, *Lower*).

In control Cpx2 KO neurons, Cpx3 and Cpx4 mRNA levels were more than 25- and 1,000-fold lower, respectively, than Cpx1 mRNA levels (Fig. 3). Compared with the Cpx2 KO, the DKO surprisingly decreased Cpx3 mRNA levels approximately twofold, but did not affect Cpx4 mRNA levels. In contrast, the Cpx1/2 DKD significantly increased the Cpx3 and Cpx4 mRNA levels, strikingly even in the presence of the Cpx1/2 DKO (Fig. 3). These changes suggest that the Cpx1/2 DKD causes a compensatory increase in Cpx3 and Cpx4 mRNA levels that may account, at least in part, for the increase in mEPSC frequency observed in the Cpx1/2 DKD neurons.

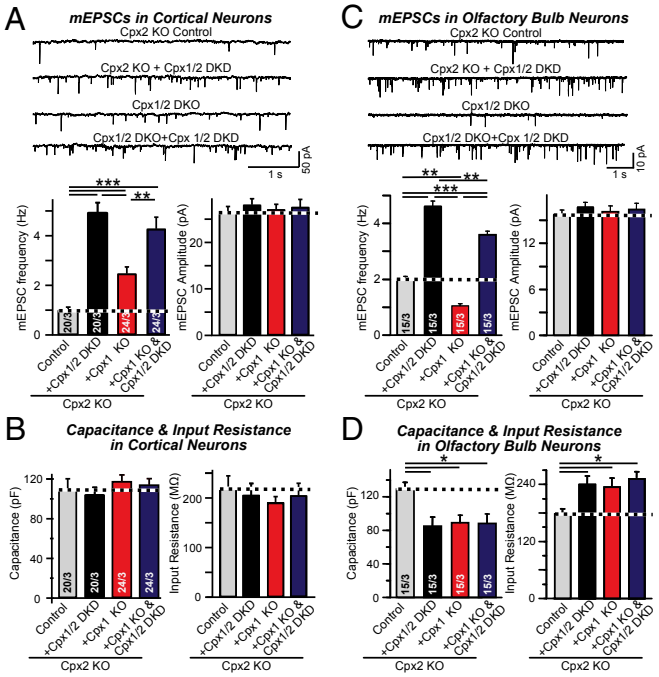

Fig. 2. Systematic comparison of complexin DKO vs. DKD phenotypes in cultured cortical and olfactory bulb neurons. (*A*) Sample traces (*Upper*) and summary graphs of the frequency (*Lower Left*) and amplitude (*Lower Right*) of spontaneous miniature excitatory postsynaptic currents (mEPSCs), recorded in cortical neurons cultured from Cpx2 single KO mice or from Cpx1/2 DKO mice without or with introduction of the Cpx1/2 DKD. (*B*) Summary graphs of the capacitance (*Left*) and input resistance (*Right*) of cortical neurons obtained as described for *A*. (*C* and *D*) Same as *A* and B, but measured in neurons cultured from olfactory bulb. Data shown are means ± SEM; numbers of neurons/independent cultures analyzed are depicted in bars. Statistical significance was assessed by Student *t* test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

Effect of Anchoring Cpx1 to the Plasma Membrane. It is striking that increases in Cpx3 and Cpx4 levels are associated with unclamping of spontaneous release in Cpx1/2 DKD neurons, as we previously found that Cpx3 is unable to support clamping of release, even though it fully activates exocytosis (17). Cpx3 and Cpx4 differ from Cpx1 and Cpx2 in containing a C-terminal "CAAX" box that causes their isoprenylation, thereby attaching Cpx3 and Cpx4 to the plasma membrane (35). We thus asked whether simple addition of the C-terminal Cpx3 sequence to Cpx1 would selectively alter the clamping activity of Cpx1.

Indeed, we found that Cpx1 containing a C-terminal Cpx3 isoprenylation sequence ($Cpx1^{CAIM}$) was only partly able to rescue the unclamping phenotype of Cpx1/2 DKD neurons, even though it fully rescued the other Cpx1/2 DKD phenotypes (Fig. 4). Thus, simply attaching Cpx1 to the plasma membrane alters its clamping function.

Overexpression of Complexin Clamping Mutants by Itself Increases Spontaneous Release. The results of the mRNA measurements raise the possibility that increased expression of Cpx3 induced by the DKD may increase spontaneous neurotransmitter release. To address this possibility, we examined the effect of overexpressing Cpx1, Cpx3, and various Cpx1 mutants in wild-type neurons, and compared their effects with those of the DKD (Fig. 5 *A* and *B*). In these experiments, we not only analyzed Cpx1 mutants that we previously found to block either both vesicle priming and clamping ($Cpx1^{1\text{-}86}$), or only vesicle clamping ($Cpx1^{poorclamp}$) (15, 17), but also the Cpx1 mutant containing a C-terminal isoprenylation signal ($Cpx1^{CAIM}$), as described above (Fig. 4). We then measured both spontaneous mEPSCs (Fig. 5*A*) and evoked EPSCs (Fig. 5*B*).

Strikingly, we found that overexpression of wild-type Cpx3 in wild-type neurons produced an approximately threefold increase in mEPSC frequency (Fig. 5*A*), and an almost twofold increase in evoked EPSC amplitudes (Fig. 5*B*). Overexpression of wild-type Cpx1 had no effect on either parameter. Overexpression of $Cpx1^{poorclamp}$ and $Cpx1^{CAIM}$ mutants increased the mEPSC frequency twofold to threefold, but did not change the evoked EPSC amplitude, whereas overexpression of the $Cpx1^{1\text{-}86}$ mutant had no effect on either parameter (Fig. 5). Thus, $Cpx3^{WT}$, $Cpx1^{CAIM}$, and $Cpx1^{poorclamp}$ serve as dominant negatives of spontaneous release, whereas the $Cpx1^{1\text{-}86}$ mutant does not, even though it is also unable to rescue the clamping phenotype in complexin-deficient neurons (17).

"Unclamping" of Spontaneous Release Is Not Always Associated with Increases in Cpx3 mRNA Levels. Our results raise the question whether Cpx1 mutants that do not rescue the increased mEPSC frequency in DKD neurons but do rescue evoked release, and that when overexpressed directly cause an increase in mEPSC frequency, do so by increasing Cpx3 expression. To test this

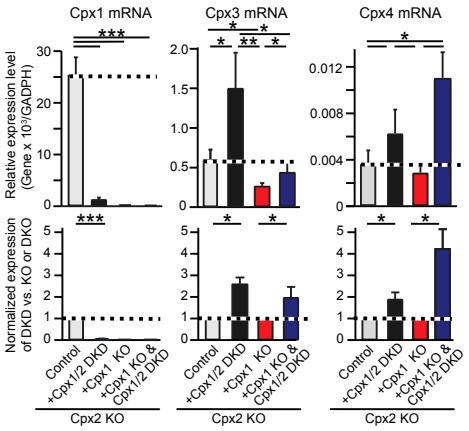

Fig. 3. The complexin-1/2 DKD but not the complexin-1/2 DKO produces a compensatory increase in Cpx3 and Cpx4 mRNA levels. Measurements of complexin mRNA levels in cultured cortical neurons from Cpx2 KO mice without or with concomitant Cpx1 KO, and with infection with control or complexin-1/2 DKD lentivirus. Neurons were infected at DIV4, and analyzed at DIV15. Data were obtained by quantitative RT-PCR and are expressed relative to GAPDH mRNA levels measured as internal controls. Absolute levels (relative to those of GAPDH mRNA measured in the same sample) are shown in the upper graphs, and relative levels (normalized to the non-DKD sample for each pair of cultured neurons obtained from the same genotype) in the lower graphs. Data shown are means ± SEM ($n = 4$ independent culture experiments); statistical significance was analyzed by Student t test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

question, we measured Cpx1, Cpx2, Cpx3, and Cpx4 mRNA levels in wild-type control neurons and neurons expressing the DKD shRNAs without or with coexpression of wild-type or mutant Cpx1. We found that wild-type and mutant Cpx1 constructs were overexpressed at similar levels (~30–40 fold; Fig. 5*C*), but left the Cpx2 level intact. However, we observed that the increase in Cpx3 and Cpx4 mRNA induced by the DKD was reversed not only by wild-type Cpx1, but also by the three different Cpx1 mutants tested, including the Cpx1^{poorclamp} mutant, which produces an increase in spontaneous release (Fig. 5*C*). Thus, the increase in spontaneous release in DKD neurons is unlikely to be caused by the induction of Cpx3 and Cpx4 expression because this increase persists after expression of the Cpx1^{poorclamp} mutant even though that mutant reverses the induction of Cpx3 and Cpx4 expression.

Discussion

Complexin function in mammalian neurons has been largely studied by two different approaches, constitutive DKO of Cpx1 and Cpx2 (4, 13, 16), and acute DKD of Cpx1 and Cpx2 (14, 15, 17). DKO neurons were largely analyzed in autapses, whereas DKD neurons were examined in high-density cultures. Overall, the two approaches resulted in similar conclusions (4, 13–17), with two important differences: the DKO approach did not reveal a decrease in the size of the RRP, and did not detect an increase in spontaneous mEPSC events, whereas the DKD approach invariably identified both. Although a genetic KO can generally be considered more reliable than a KD because of the massive off-target effects of KDs, the DKD results were confirmed in fly and worm neurons carrying complexin mutations (5, 6, 18, 27–30).

As a result of the different conclusions emerging from the complexin DKO and DKD studies, the function of complexin in vesicles priming and in clamping spontaneous exocytosis has remained unclear. To address this issue, we have now directly compared, in the same experiment and using the same preparation, the phenotypes produced by the DKD and DKO approaches. To maximize the phenotypic analyses, we also performed all DKO and DKD experiments in combination, and additionally examined two different types of cultured neurons, cortical and olfactory bulb neurons.

Our data show that for release evoked by single isolated action potentials (to measure Ca^{2+} triggering of exocytosis) and by hypertonic sucrose (to measure the size of the RRP), the DKO and DKD approaches produced nearly identical phenotypes in both cultured cortical and olfactory bulb neurons (Fig. 1). In particular, the size of the RRP was similarly decreased approximately twofold in DKD and DKO neurons without a significant change in the kinetics of sucrose-induced release between DKD, DKO, and wild-type neurons. This result confirms an essential and significant role for complexin in vesicle priming consistent with studies in invertebrate neurons (28, 29).

In contrast to evoked release, we observed quite distinct effects of the DKO and DKD on spontaneous exocytosis (Fig. 2). Whereas the DKD invariably increased spontaneous release approximately fourfold (unclamping), the DKO caused a ~2.5-fold increase in spontaneous release in cortical neurons, but an approximately twofold decrease in spontaneous release in olfactory bulb neurons (Fig. 2). In both types of neurons, the DKO/DKD combination produced a phenotype identical to that of the DKD. These data suggest that, although complexin has a role in clamping exocytosis in some contexts, this role—different from the general function of complexin in priming vesicles and in activating Ca^{2+} triggering of exocytosis—is not invariably present. It should be noted here that the overall degree of unclamping, i.e., activation of exocytosis by removal of complexins, is very small compared with the activation of exocytosis produced by Ca^{2+} (36).

We hypothesized that a potential compensatory increase of Cpx3 or Cpx4 expression might occur in constitutive DKO neurons. Surprisingly, however, we detected no evidence for a compensatory change in constitutive DKO neurons, but found an increase in Cpx3 and Cpx4 mRNA levels in DKD neurons that

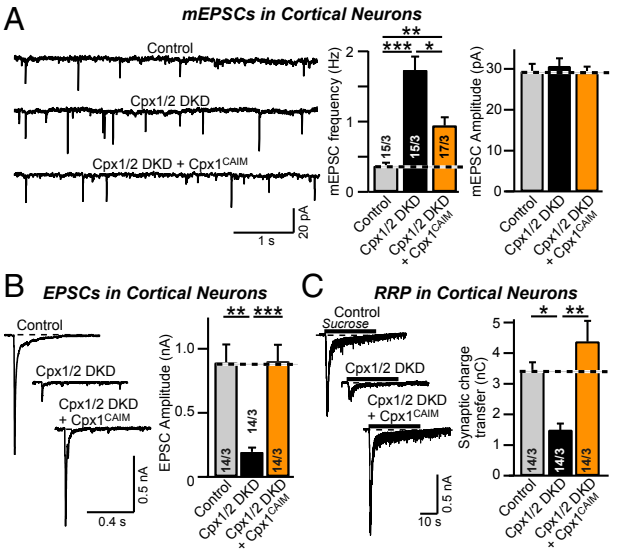

Fig. 4. Lipid anchoring complexin-1 partly blocks the clamping function of Cpx1 but has no effect on the activating functions of Cpx1. (*A*) Sample traces (*Left*) and summary graph of the frequency and amplitude (*Right*) of mEPSCs monitored in control neurons, Cpx1/2 DKD neurons, and Cpx1/2 DKD neurons that overexpress mutant Cpx1 containing a C-terminal "CAIM" sequence (Cpx1^{CAIM}) that causes isoprenylation and membrane attachment of complexins. (*B*) Sample traces of action potential-evoked EPSCs (*Left*) and summary graph of the EPSC amplitude (*Right*) monitored in cortical neurons obtained as described for *A*. (*C*) Sample traces of sucrose-evoked EPSCs (*Left*) and summary graphs of the charge transfer induced by hypertonic sucrose (*Right*) recorded in cortical neurons obtained as described for *A*. Release was triggered by a 30-s application of 0.5 M sucrose, and the synaptic charge transfer was integrated over 30 s. All data shown are means ± SEM; numbers of neurons/independent cultures analyzed are depicted in the bars. Statistical significance was analyzed by Student t test, with *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

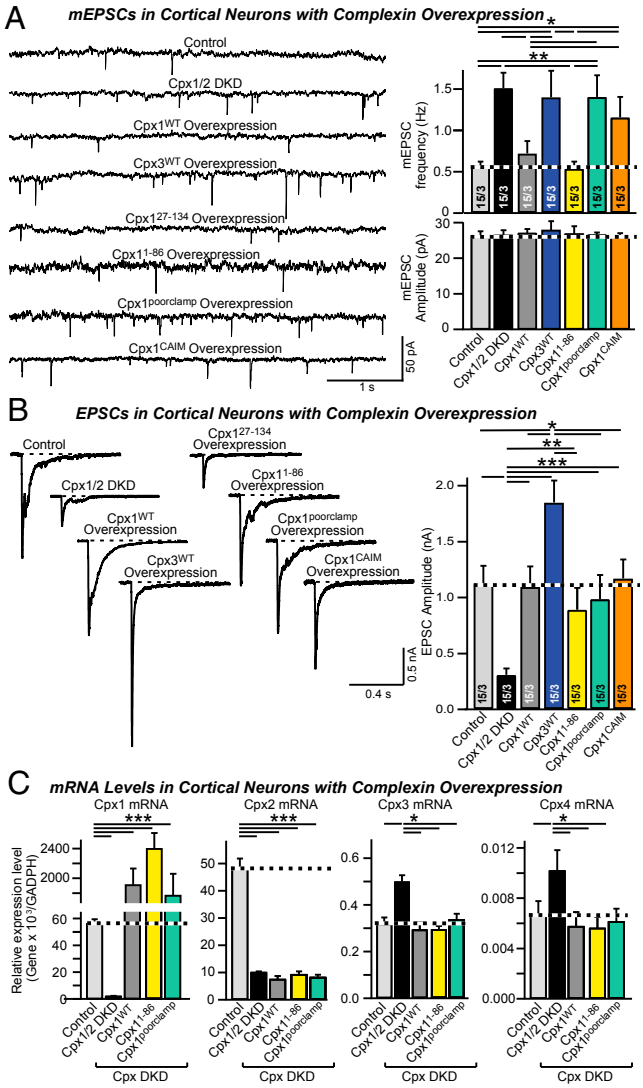

Fig. 5. Mechanism of clamping by complexins: Overexpression of Cpx1 mutants that unclamp mini release increases the mEPSC frequency, but these Cpx1 mutants do not act by increasing Cpx3 expression. (*A*) Sample traces (*Left*) and summary graph of the frequency and amplitude (*Right*) of mEPSCs monitored in control neurons, complexin KD neurons (Cpx1/2 DKD), and neurons after overexpression of wild-type Cpx1 ($Cpx1^{WT}$), wild-type Cpx3 ($Cpx3^{WT}$), C-terminally truncated Cpx1 ($Cpx1^{1-86}$), the Cpx1 poorclamp mutant ($Cpx1^{poorclamp}$), or the Cpx1 lipid-anchor mutant ($Cpx1^{CAIM}$). Experiments were carried out in cultured cortical neurons. (*B*) Sample traces (*Left*) and summary graph of the amplitude (*Right*) of action potential-evoked EPSCs monitored in cultured cortical neurons treated as described for *A*. (*C*) Measurements of Cpx1, Cpx2, Cpx3, and Cpx4 mRNA levels in control neurons and in Cpx DKD neurons overexpressing either GFP (Cpx1/2 DKD), wild-type Cpx1 ($Cpx1^{WT}$), C-terminally truncated Cpx1 ($Cpx1^{1-86}$), or the Cpx1 poorclamp mutant ($Cpx1^{poorclamp}$). Experiments were performed in cultured cortical neurons infected at DIV4, and analyzed at DIV15; mRNA levels are expressed relative to those for GAPDH measured in the same samples. Data shown are means ± SEM. For *A* and *B*, numbers of neurons/independent cultures analyzed are depicted in the bars; for *C*, $n = 3$ independent culture experiments. Statistical significance was analyzed by Student *t* test, with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

was observed even when the DKD was applied to DKO neurons (Fig. 3). This increase is surprising because compensatory effects would be expected more readily for constitutive DKO than for acute DKD manipulations. Moreover, the DKD should theoretically not be able to knock down anything in the DKO because Cpx1 and Cpx2 are no longer expressed. The mRNA measurements thus suggest that the lack of an unclamping phenotype in DKO neurons cannot be explained by compensatory changes in another complexin isoform, although these results do not rule out other types of compensation.

The results of the mRNA measurements (Fig. 3) prompted the hypothesis that the increase in Cpx3 and Cpx4 could be responsible for the unclamping phenotype in DKD neurons, a hypothesis that we examined by three approaches. First, we examined whether Cpx3 is inherently prone to activating spontaneous release because Cpx3 is anchored to the plasma membrane via a lipid modification (Fig. 4) (30); second, we tested whether overexpression of Cpx3 could increase the mini frequency (Fig. 5 *A* and *B*), and third, we investigated whether the increase in Cpx3 and Cpx4 mRNA levels is also present in DKD neurons in which mutant complexins were expressed that correct impaired evoked release but do not clamp spontaneous release (Fig. 5*C*).

First, we found that adding a lipid anchor to Cpx1, which is thought to localize it to the plasma membrane, impaired the ability of Cpx1 to "clamp" spontaneous release without interfering with its activating functions (Fig. 4). This result demonstrated that the apparent clamping functions of Cpx1 but not its priming and Ca^{2+}-triggering functions are dependent on its localization consistent with previous studies (30).

Second, we observed that overexpression of Cpx3 or of clamping mutants of Cpx1 in wild-type neurons cause an increase in mini frequency even without the complexin DKD (Fig. 5). Thus, the unclamping effect of these mutants is dominant negative. This result seems to support the notion that the compensatory increase in Cpx3 levels in DKD neurons (Fig. 3) may unclamp mini release, but does not rule out the alternative possibility that the mutant complexins directly interfere with the release machinery to unclamp release.

We differentiated between these two possibilities in the third set of test experiments. We found that rescue of the DKD neurons using the $Cpx1^{poorclamp}$ mutant (which reversed the impairment in evoked release but did not ameliorate the increase in spontaneous release) also reversed the increase in Cpx3 mRNA levels in these neurons (Fig. 5*C*). This result implies that the compensatory increase in Cpx3 mRNA in DKD neurons is related to the loss of the activating functions of complexin, and can be reversed by simply restoring only the activating function. Thus, unclamping cannot be solely due to an increase in Cpx3 mRNA levels, suggesting that there is a direct unclamping effect of mutant complexin.

An incidental finding of our study is that simply targeting Cpx1 to the plasma membrane by adding to its C terminus a lipid-anchor sequence that is normally present in Cpx3 and Cpx4 selectively blocks its clamping function, but not its Ca^{2+}-triggering and priming functions (Fig. 4). Moreover, simple overexpression of lipid-anchored Cpx1 unclamps spontaneous release, whereas overexpression of wild-type Cpx1 has no effect (Fig. 5*A*). These data complement observations that complexin when localized to synaptic vesicles clamps release (12, 30).

Viewed together, our results establish that complexin performs basic functions as a priming factor that maintains a normal-sized RRP, and as an activating factor that allows fast Ca^{2+} triggering of release by synaptotagmin. These functions are likely separate activities of complexin because they depend on distinct complexin sequences (15, 17, 18). However, our results suggest that the clamping function of complexin in mammalian neurons, despite its evolutionary conservation in *Drosophila* (5, 6, 27, 29, 37), is not general but highly dependent on the biological context.

At first sight, the unclamping of spontaneous release observed in DKD neurons resembles an off-target effect, because this unclamping is observed even in DKO neurons lacking Cpx1 and Cpx2. However, the specific increase in Cpx3 and Cpx4 mRNA levels in DKD neurons and the rescue of this increase by Cpx1 mutants that do not rescue the clamping phenotype are difficult to explain as an off-target effect (Fig. 5*C*). Even more strikingly, overexpression of clamping-deficient mutant Cpx1 in wild-type neurons by itself causes unclamping, which also cannot

be explained by an off-target effect. Thus, off-target effects alone cannot account for the unclamping phenotype of the DKD neurons, and we currently cannot reconcile these different observations.

A related question regards the observation that the DKO decreased clamping of spontaneous release ("unclamped") in cortical neurons, but increased clamping in olfactory bulb neurons—how is this possible? Here, an effect of complexin on other types of exocytosis may have to be considered, as exemplified by the requirement for complexin in IGF1 exocytosis (32) and in postsynaptically induced NMDA receptor-dependent long-term potentiation (38). The decrease in IGF1 secretion in the DKO neurons may have altered neuronal maturation and thereby caused a reduction of mEPSC frequency in the DKO neurons, whereas the acute decrease in IGF1 secretion in the DKD neurons may have set in too late developmentally to have such an effect. At least some of the synaptic phenotypes that have been observed for DKO and DKD neurons may thus be an indirect consequence of the loss of other forms of complexin-dependent types of regulated exocytosis (32, 38). In other words, different phenotypes may be due to diverse function of complexin in different types of exocytosis that cause indirect effects. This possibility is supported by the universal presence of complexin in most animal cells throughout the body (2), and will require understanding the full complement of complexin functions for confirmation.

In summary, we here confirm a general role for complexin in synaptic vesicle priming in addition to Ca^{2+} triggering of exocytosis, and demonstrate that the apparent function of complexin in clamping spontaneous synaptic vesicle exocytosis is highly dependent on the experimental context and on the localization of complexin. Paramount among the crucial questions raised by these results is how complexin might function in priming, and how biologically significant the role of complexin in spontaneous release is.

Methods

Cpx1/Cpx2 DKO Mice. Cpx1/Cpx2 DKO mice (4) were generated and genotyped as described (4). All animal procedures were performed in accordance with Stanford animal use rules and the requisite approvals of animal use committees.

Culture and Lentiviral Infection of Neurons. Culture and lentiviral infection of neurons were obtained from mouse cortex and olfactory bulb as described (15, 39), and were infected with lentiviruses using standard procedures (14, 15, 17) (see *SI Methods*).

mRNA Measurements. mRNA measurements were performed with RNA isolated from cultured neurons at 15 d in vitro (DIV15) using the RNAqueous kit (Ambion) following the manufacturer's directions. Gene-specific probes were obtained from IDT. RT-PCR reactions were set up in triplicates for each condition with 150 ng of total RNA using the LightCycler 480 reagent kit (Roche). Reactions were run and analyzed using a 7900HT Fast RT-PCR instrument (Applied Biosystems) with GAPDH as internal control.

Electrophysiological Recordings. Electrophysiological recordings were performed in whole-cell patch-clamp mode using concentric extracellular stimulation electrodes as described (15). For experimental details, see *SI Methods*.

Statistical Analyses. Statistical analyses were performed with Student *t* tests comparing test to control samples analyzed in the same experiments.

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