Cellular/Molecular

Complexin Controls Spontaneous and Evoked Neurotransmitter Release by Regulating the Timing and Properties of Synaptotagmin Activity

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Neurotransmitter release following synaptic vesicle (SV) fusion is the fundamental mechanism for neuronal communication. Synaptic exocytosis is a specialized form of intercellular communication that shares a common SNARE-mediated fusion mechanism with other membrane trafficking pathways. The regulation of synaptic vesicle fusion kinetics and short-term plasticity is critical for rapid encoding and transmission of signals across synapses. Several families of SNARE-binding proteins have evolved to regulate synaptic exocytosis, including Synaptotagmin (SYT) and Complexin (CPX). Here, we demonstrate that *Drosophila* CPX controls evoked fusion occurring via the synchronous and asynchronous pathways. $cpx^{-/-}$ mutants show increased asynchronous release, while CPX overexpression largely eliminates the asynchronous component of fusion. We also find that SYT and CPX coregulate the kinetics and Ca²⁺ co-operativity of neurotransmitter release. CPX functions as a positive regulator of release in part by coupling the Ca²⁺ sensor SYT to the fusion machinery and synchronizing its activity to speed fusion. In contrast, $syt^{-/-}$; $cpx^{-/-}$ double mutants completely abolish the enhanced spontaneous release observe in $cpx^{-/-}$ mutants alone, indicating CPX acts as a fusion clamp to block premature exocytosis in part by preventing inappropriate activation of the SNARE machinery by SYT. CPX levels also control the size of synaptic vesicle pools, including the immediate releasable pool and the ready releasable pool— key elements of short-term plasticity that define the ability of synapses to sustain responses during burst firing. These observations indicate CPX regulates both spontaneous and evoked fusion by modulating the timing and properties of SYT activation during the synaptic vesicle cycle.

Introduction

Synaptic vesicles (SV) can fuse either spontaneously, or wait in a primed and fusion-competent state for evoked Ca²⁺ influx to trigger activation of the SNARE complex fusion machinery (Söllner et al., 1993; Chen et al., 1999; Vrljic et al., 2010). The SNARE proteins synaptobrevin/VAMP, syntaxin, and SNAP-25 assemble into an α -helical coiled-coil structure that brings the SV and plasma membrane in close proximity for fusion (Sutton et al., 1998). During evoked release, vesicles can fuse immediately through a synchronous pathway, or with a small delay via an asynchronous release mechanism. The synaptic vesicle protein synaptotagmin 1 (SYT) functions as the synchronous Ca²⁺ sen-

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sor and engages lipids and SNAREs following Ca²⁺ influx (Geppert et al., 1994; Yoshihara and Littleton, 2002; Choi et al., 2010; Kochubey and Schneggenburger, 2011). The SNARE binding protein complexin (CPX) also regulates both evoked fusion and spontaneous release (minis) (Reim et al., 2001; Giraudo et al., 2006; Huntwork and Littleton, 2007; Maximov et al., 2009; Yang et al., 2010; Hobson et al., 2011; Martin et al., 2011). Although CPX and SYT are key regulators of neurotransmitter release, the mechanisms by which SYT and CPX act to orchestrate fusion are still unclear. SYT contains an N-terminal synaptic vesicle transmembrane anchor and a cytoplasmic region containing two Ca²⁺-binding C2 domains (Perin et al., 1990). Studies of SYT in Drosophila indicate Ca2+ binding to the C2B domain triggers synchronous fusion (Mackler et al., 2002; Yoshihara et al., 2010), while Ca²⁺ binding to the C2A domain is required to suppress asynchronous release (Yoshihara et al., 2010) and help facilitate synchronous fusion (Striegel et al., 2012). In contrast to the Ca²⁺-binding activity of SYT, CPX lacks Ca²⁺-binding sites and is a small cytoplasmic protein with a central α -helix that interacts with the SNARE complex (McMahon et al., 1995; Pabst et al., 2000; Bracher et al., 2002). SNARE binding by CPX functions to facilitate synaptic vesicle release (Reim et al., 2001; Xue et al., 2009; Cho et al., 2010; Xue et al., 2010) and allows CPX to act as a fusion clamp to prevent premature exocytosis in the absence of Ca²⁺ (Huntwork and Littleton, 2007; Maximov et al., 2009). How CPX and SYT coordinate whether SV fusion occurs spon-

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taneously or during action potential-triggered evoked release is still unclear. Likewise, little is understood concerning how these proteins regulate aspects of presynaptic output during burst firing that underlies short-term plasticity. Here, we describe experiments where release kinetics and short-term plasticity in SYT and CPX loss-of-function mutants and overexpression strains were measured using high-fidelity current recordings of postsynaptic responses in voltage-clamp at the neuromuscular junction (NMJ) of *Drosophila* larvae. The *Drosophila* glutamatergic NMJ provides a model synapse that shares basic molecular components of synaptic transmission with those found at most vertebrate synapses, allowing a detailed analysis of how neuronalspecific SNARE-binding proteins regulate multiple components of information transfer at glutamatergic synapses.

Materials and Methods

Drosophila *stocks and genetics.* The homozygous cpx^{SH1} deletion allele was used as the genotype for $cpx^{-/-}$ null mutants, while a precision excision was used as the genetic control. *Syt 1* null mutants were generated by crossing syt^{N13} and syt^{AD4} alleles. Overexpression of UAS-CPX and UAS-SYT was driven by pan-neuronal *elav*-Gal4 (C155). Both UAS constructs rescue the null phenotypes for cpx and syt mutants (Yoshihara and Littleton, 2002; Huntwork and Littleton, 2007), respectively. Flies of either sex were analyzed.

Western blot analysis and immunocytochemistry. Western blots were performed using standard laboratory procedures. Nitrocellulose membranes were probed with anti-CPX or anti-Synaptogyrin antibodies. Antiserum to CPX was used at a dilution of 1:10,000. Antiserum to Synaptogyrin was used at a dilution of 1:1000. The probes were detected using IRDye 680-conjugated goat anti-rabbit IgG at a dilution of 1:3000 (LI-COR Biosciences). Visualization and quantification was done using the LI-COR Odyssey Imaging System (LI-COR Biosciences). Immunocytochemistry was performed on third instar larvae as previously described (Barber et al., 2009) but without the secondary labeling step. Affinity purified antibodies prepared against anti-GluRIII (rabbit antibody raised against GluRIII C-terminal peptide - QGSGSSSGSNNAGRGEKEARV) and nc82 anti-BRP (mouse monoclonal antibody from Developmental Studies Hybridoma Bank) were directly labeled with Alexa Fluor 488 and 546, respectively (Invitrogen). Both antibodies were used at 1 μ g/1 ml. Confocal stacks of muscle 4 NMJ were captured using a laser-scanning confocal microscope (Pascal; Carl Zeiss) with a 40× NA 1.3 Plan Neofluar oil-immersion lens (Carl Zeiss).

Electrophysiology. Postsynaptic currents from the specified genotypes were recorded at segment A3 of ventral longitudinal muscle 5 or 6 as indicated in third instar larvae using two-electrode voltage clamp with a -80 mV holding potential in modified HL3 solution (in mM: 10 NaHCO₃, 5 KCl, 4 MgCl, 5 HEPES, 70 NaCl, 5 Trehalose, 115 Sucrose, pH 7.2). Final Ca²⁺ concentration was adjusted to the desired level indicated in each figure legend. Data acquisition and analysis was performed using Axoscope 9.0 and Clampfit 9.0 software (Molecular Devices). Quantal content was estimated by dividing the current integral of nerve-evoked currents by the current integral from individual quanta as previously described (Delgado et al., 2000; Barber et al., 2009). Motor nerves innervating the musculature were severed and placed into a suction electrode so action potential stimulation could be applied at the indicated frequencies using a programmable stimulator (Master8, AMPI).

EM analysis. Electron microscopy and quantification of SV distribution were performed as previously described (Akbergenova and Bykhovskaia, 2009), with the exception that sections were cut at 60 nm thickness and stained with 2% uranyl acetate.

Data analysis and statistics. Electrophysiology analysis was performed using Clampfit 9.0 software (Molecular Devices). Statistical analysis and graphs were performed using Origin Software (OriginLab Corporation). Statistical significance was determined using a two-tailed Student's *t* test. For all data, error bars represent SEM.

Results

CPX regulates spontaneous and evoked fusion rates

Our previous studies revealed that cpx null mutants $(cpx^{-/-})$ show a dramatic elevation in spontaneous fusion at Drosophila NMJs (Huntwork and Littleton, 2007). To further examine how CPX regulates release, we characterized spontaneous release parameters in $cpx^{-/-}$, $cpx^{+/-}$, and CPX overexpression lines that scale the levels of CPX from 0 to \sim 250% of control as shown by Western analysis (Fig. 1A). Manipulations of CPX levels did not alter basic synaptic substructure visualized by immunocytochemistry, as active zone apposition to glutamate receptor fields and glutamate receptor cluster size was similar to controls (data not shown). As shown in Figure 1B, spontaneous fusion events from single SVs are easily distinguished from basal noise in the absence of stimulation. $cpx^{-/-}$ null mutants displayed a ~40fold increase in spontaneous frequency (80.6 \pm 9.3 Hz) compared with controls $(2.6 \pm 0.2 \text{ Hz})$ at muscle 6 NMJs. The dramatic increase in spontaneous release was not reduced in 0 mM extracellular Ca^{2+} (Huntwork and Littleton, 2007) or in the presence of Cd²⁺, which blocks presynaptic Ca²⁺ entry (Fig. 1*D*), indicating increased spontaneous release in $cpx^{-/-}$ does not require extracellular Ca²⁺ influx. Heterozygous $cpx^{+/-}$ animals with a 50% reduction in CPX protein levels (Fig. 1A) also displayed an elevated spontaneous release frequency of 7.3 \pm 0.6 Hz (Fig. 1B,D). Overexpression of CPX presynaptically by \sim 2.4fold using the UAS-GAL4 system (Fig. 1A) reduced baseline spontaneous release to 1.5 ± 0.1 Hz (Fig. 1*B*,*D*), indicating CPX is rate-limiting as a fusion clamp at Drosophila NMJs. The increase in spontaneous release observed at $cpx^{-1/}$ muscle 6 NMJs made it difficult to analyze single unitary fusion events. To bypass summation effects at muscle 6, we examined neighboring muscle 5 NMJs, a synapse with \sim 4-fold fewer release sites. Similar to muscle 6, $cpx^{-/-}$ increased spontaneous release ~40-fold (16 ± 2 Hz in $cpx^{-/-}$ compared with 0.5 ± 0.1 Hz in controls, Fig. 1C,D). The reduced frequency at muscle 5 allowed a detailed quantal analysis of isolated single SV fusion events. Quantal size and individual vesicle release kinetics, including the rise and decay times, were not altered in $cpx^{-\prime -}$ (Fig. 1*E*, *F*), indicating CPX does not modulate SV fusion pore dynamics for quantal glutamate discharge, but rather controls the overall rate of spontaneous fusion events.

In addition to its role as a fusion clamp, CPX is required for normal levels of evoked release (Reim et al., 2001; Huntwork and Littleton, 2007). To determine if CPX is rate-limiting in its role as a positive regulator of release, we assayed evoked properties in $cpx^{-/-}, cpx^{+/-}$, and CPX overexpression lines. We observed that $cpx^{-/-}$ has a ~60% reduction in evoked release amplitude, while overexpression of CPX enhanced evoked responses by $\sim 25\%$ (Fig. 2*A*). Expressing a transgenic UAS-cpx in the $cpx^{-/-}$ background with the neuronal drive elav-GAL4 fully rescued release defects in the mutant (Fig. 2A), suggesting CPX functions solely in the presynaptic compartment in Drosophila to control neurotransmitter release. Heterozygous $cpx^{+/-}$ lines reduced release by ~15% (214 \pm 20 nA in $cpx^{+/-}$ compared with 260 \pm 20 nA in controls, data not shown), indicating CPX is rate-limiting for both spontaneous and evoked release. To examine whether the effect of CPX on evoked release was mediated by shifting Ca²⁺ affinity versus altering Ca^{2+} co-operativity, we measured the Ca^{2+} dependence of fusion in $cpx^{-/-}$ and CPX overexpression lines (Fig. 2B). Surprisingly, manipulations of CPX levels altered Ca²⁺ co-operativity, with a reduction in the Ca²⁺ dependence of release from 3.3 in controls to 2.1 in $cpx^{-/-}$ mutants, similar to



Figure 1. CPX levels control the rate of spontaneous synaptic vesicle fusion. *A*, Quantification of CPX levels from Western analysis of independent samples of control, $cpx^{-/-}$, $cpx^{+/-}$, and CPX overexpression lines. A representative Western blot from head lysates is shown on the right. Antibodies to the synaptic vesicle protein synaptogyrin were used as a loading control. *B*, Postsynaptic current recordings of spontaneous release at muscle 6 synapses in control (black), $cpx^{-/-}$ null mutants (red), $cpx^{+/-}$ heterozygotes (green), and animals overexpressing UAS-CPX driven by *elav*-GAL4 (0E-CPX, blue). This genotypic color code is maintained throughout the figures. Mean mini frequency in the genotypes are as follows: control, 2.6 ± 0.2 Hz; $cpx^{-/-}$, 80.6 ± 9.3 Hz; $cpx^{+/-}$, 7.3 ± 0.6 Hz; 0E-CPX, 1.5 ± 0.1 Hz. Student's *t* test revealed significant differences between control and $cpx^{-/-}$ (p < 0.01), and control and 0E-CPX (p < 0.01). *C*, Recordings at muscle 5 in control and $cpx^{-/-}$ synapses. Mean mini frequency in the genotypes are as follows: control, 0.5 ± 0.1 Hz; $cpx^{-/-}$, 16 ± 2 Hz. Student's *t* test revealed significant of average mini frequency in each condition on a semi-logarithmic plot. Addition of 2 mm Cd²⁺ did not decrease mini release at $cpx^{-/-}$ synapses. *E*, Histograms of mEPSC peak amplitudes in control (top) and $cpx^{-/-}$. The histogram of the spontaneous miniature amplitude was fitted with a single Gaussian distribution in control synapses and with a multiple Gaussian distribution. *F*, mEPSC analysis in control (black) and $cpx^{-/-}$ synapses. Average traces were obtained by summing all mEPSC events under the first peak distribution ± 2 SD. The normalized cumulative charge transferred in control and $cpx^{-/-}$ synapses. Error bars, SEM.

co-operativity changes found in *syt*^{-/-} mutants (Littleton et al., 1994; Yoshihara and Littleton, 2002). In contrast, CPX overexpression enhanced Ca²⁺ co-operativity from 3.3 to 3.9. These data indicate the Ca²⁺ dependence of release critically depends on CPX levels, suggesting a potential interplay between CPX and the Ca²⁺ sensor SYT in triggering evoked release.

CPX enhances the speed of evoked quantal release

The EPSC recorded in the muscle after evoking an action potential represents the macroscopic summation of multiple individual miniature EPSCs (mEPSCs) that occur across many release sites. After nerve stimulation, guantal release is activated with a certain probability during the time window of elevated intraterminal Ca²⁺. Evoked SV fusion occurs through a rapid SYT-regulated synchronous fusion pathway and a slower SYTindependent asynchronous pathway (Geppert et al., 1994; Yoshihara and Littleton, 2002; Xu et al., 2012). To determine whether CPX differentially regulates these distinct pathways, we examined synchronous and asynchronous release in cpx⁻ mutants and CPX overexpression lines (Fig. 2C-G). Comparing the quantal release rate normalized to the maximum response (Fig. 2C), evoked vesicle release was slowed in $cpx^{-/-}$ mutants (mean time constant = 12.9 ± 1.2 ms) and enhanced by CPX overexpression (mean time constant = 10.6 ± 1.5 ms) compared with controls (mean time constant = 11.9 ± 1.3 ms). In addition, $cpx^{-/-}$ mutants reduced the amount of synchronous release, while increasing the asynchronous component of vesicle fusion (Fig. 2E, F). In contrast, CPX overexpression almost completely suppressed all asynchronous fusion (Fig. 2E, F). The average asynchronous component in controls was $5.0 \pm 1.5\%$, compared with $15 \pm 2.9\%$ in $cpx^{-/-}$ and $0.7 \pm 0.4\%$ in CPX overexpression animals. The time constant for the kinetics of the slow component of release was not affected by CPX levels (Fig. 2G), indicating CPX controls the relative amount of fusion occurring via the synchronous or asynchronous pathway. Part of the slower kinetics of release observed in the evoked responses in $cpx^{-/-}$ mutants could in theory be associated with abnormal postsynaptic responses due to the high amount of spontaneous neurotransmitter release. To observe whether there is more miniature fluctuation during the evoked responses in $cpx^{-/-}$, we recorded evoked responses at the muscle 5. Control synapses showed a synchronous evoked response with lower amplitude than muscle 6, in agreement with fewer release sites at this muscle. However, $cpx^{-/-}$ synapses not only show a reduction in the evoked amplitude compared with controls, but also an increase in the number of miniature fluctuations during a 50 ms window following release. The mean number of distinguishable events in control (1.05 ± 0.01) is far less than that observed in $cpx^{-/-}$ (9.1 \pm 0.5; Student's t test, p < 0.001). The instantaneous frequency of events evoked in $cpx^{-/-}$ (182 ± 10 Hz) surpasses by tenfold the rate of spontaneous events observed at rest in the same synapses



Figure 2. CPX levels control the rate of evoked synaptic vesicle fusion. *A*, Left, Representative EPSCs recorded in the presence of 2 mm external Ca²⁺ at control (black), $cpx^{-/-}$ (red), and OE-CPX synapses (blue). Mean amplitudes in the genotypes are as follows: control, 260 ± 20 nA; $cpx^{-/-}$, 100 ± 12 nA; rescue, 274 ± 30 nA; OE-CPX, 320 ± 30 nA. Student's *t* test revealed significant differences between control and $cpx^{-/-}$ (p < 0.01), rescue and $cpx^{-/-}$ (p < 0.01), and control and OE-CPX (p < 0.01). The average EPSC normalized to the maximal amplitude in each genotype is presented with a magnified time scale on the right. Right, Representative EPSCs recorded at muscle 5 in the presence of 2 mm external Ca²⁺ at control (black) and $cpx^{-/-}$ terminals (red). Mean amplitudes in the genotypes are as follows: control, 34 ± 4 nA; $cpx^{-/-}$, 12 ± 2 nA. Student's *t* test revealed significant differences between control and $cpx^{-/-}$ (p < 0.01). The average EPSC normalized to the maximal amplitude in each genotype is presented with a magnified time scale on the right. *B*, Ca²⁺ co-operativity of release is shown on a double logarithmic plot for control (black), $cpx^{-/-}$ (red), and CPX overexpression (blue). The co-operativity in the genotypes is as follows: control, 3.3 ± 0.2 ; $cpx^{-/-}$, 2.1 ± 0.1 ; OE-CPX, 3.9 ± 0.2 . Student's *t* test revealed significant differences between control and $cpx^{-/-}$ (p < 0.001) and control and OE-CPX (p < 0.03). *C*, Average normalized responses plotted on a semi logarithmic graph to display their components. Dashed lines represent the best fit for the slow component of release evaluated during the final 30 ms of the response. Mean time constants in the genotypes were not significant differences between control and $cpx^{-/-}$, $l.9 \pm 1.2$ ms; 0E-CPX, $lo \pm 1.5$ ms. *D*, Cumulative release normalized for the maximum in 2.0 mM Ca²⁺ for each genotype. Each trace was adjusted to a double exponential fit. Mean charge transf

(Fig. 1*C*,*D*). Comparative analyses of the average evoked responses between control and $cpx^{-/-}$ produce the same behavior at muscle 6. These results indicate that the slower kinetic responses observed in $cpx^{-/-}$ synapses are a consequence of an increase in delayed fusion events, not due to postsynaptic defects. In summary, the absence of CPX resulted in increased asynchronous release, while CPX overexpression largely eliminated the asynchronous component of fusion.

To further analyze the ability of CPX to regulate transmission speed, we examined how changes in Ca^{2+} concentration altered CPX-induced phenotypes. The speed of evoked release increases with elevated Ca^{2+} levels (Katz and Miledi, 1968; Felmy et al., 2003). A change in the rising face of the EPSC is indicative that the quantal release process is activated with a different probability. We compared evoked release kinetics in low (0.2 mM) versus high (2.0 mM) extracellular Ca^{2+} (Fig. 3). In control animals, the initial speed of synchronous release measured by the rise time of the evoked current was increased ~2-fold in elevated Ca^{2+} (Fig. 3*A*, *B*). Overexpression of CPX resulted in a similar twofold faster risetime in low Ca²⁺ compared with controls, suggesting CPX overexpression speeds quantal SV fusion similar to the effects observed after Ca^{2+} elevation. In high Ca^{2+} , there is no change in rise time induced by CPX overexpression, indicating release speed is saturated in these conditions. In contrast, $cpx^{-/-}$ mutants exhibit slower quantal release in both high and low Ca²⁺, indicating CPX is a critical regulator of transmission speed. Analysis of the mean rise time for evoked responses at 0.2 mM Ca^{2+} demonstrated that $cpx^{-/-}$ mutants slowed the initial rise phase to 4.5 \pm 0.4 ms, while CPX overexpression increased the rise time to 1.2 \pm 0.1 ms compared with controls (2.7 \pm 0.3 ms). We also observed that the levels of CPX regulated overall quantal release rates, in addition to the initial speed of fusion, as measured by the total rate of release (Fig. $3C_{2}D$). The mean quantal release rates in $0.2 \text{ mM} \text{ Ca}^{2+}$ measured when 90% of the total charge was transferred were 21.3 \pm 2.1 ms in control, 37.2 ± 3.72 ms in $cpx^{-/-}$, and 15.5 \pm 1.6 ms in animals overexpressing CPX. Increased CPX levels enhanced the overall quantal release rate similar to the effects of elevating external Ca^{2+} (Fig. 3D).

Use-dependent synaptic plasticity regulated by CPX

Synaptic vesicle recycling and the maintenance of specific SV pools are critical for neuronal communication during highfrequency tetanic stimulation. Three major functional pools of synaptic vesicles-the immediate releasable pool (IRP), the ready releasable pool (RRP), and the reserve pool (RP)-have been described at multiple synapses, including the Drosophila NMJ (Rizzoli and Betz, 2005). Vesicles from the IRP contribute to phasic neuronal responses and have been associated morphologically with docked synaptic vesicles (Elmqvist and Quastel, 1965; Schneggenburger et al., 1999; Delgado et al., 2000). Vesicles from the RRP drive tonic neuronal responses and are mobilized during stimulation to prevent vesicle depletion at active zones. Synaptic vesicle recycling is largely responsible for sustaining release after RRP mobilization, with a minor contribution from the RP (Heuser and Reese, 1973; Delgado et al., 2000; Zucker and Regehr, 2002). To examine how CPX regulates short-term plasticity and synaptic vesicle pools during high-frequency tetanic stimulation, we examined use-dependent changes in evoked release in 2 mM external Ca²⁺ during a 10 Hz stimulation. The initial evoked responses undergo a rapid and transient depression as vesicle pools become depleted within the first 10-15 stimuli (Fig. 4A-C). The initial fast component of depression is described by a single exponential that rapidly reaches a constant value. We attribute this early phase of synaptic depression to depletion of the IRP, with recovery through recruitment of vesicles from the RRP. The



Figure 3. CPX and Ca²⁺ increase the speed of evoked quantal release rate. *A*, Average traces of EPSCs normalized to the maximal amplitude in control (black), $cpx^{-/-}$ (red), and CPX overexpression (OE-CPX, blue) synapses in 0.2 and 2.0 mM external Ca²⁺. *B*, Quantification of average rise time in each genotype. Mean rise times at 0.2 mM Ca²⁺ are as follows: control, 2.7 ± 0.3 ms; $cpx^{-/-}$, 4.5 ± 0.4 ms; OE-CPX, 1.2 ± 0.1 ms. Student's *t* test revealed significant differences between control and OE-CPX (p < 0.01), control and $cpx^{-/-}$ (p < 0.01), control at 0.2 and 2 mM (p < 0.01), and $cpx^{-/-}$ at 0.2 and 2 mM (p < 0.01). Mean rise times at 2 mM Ca²⁺ are as follows: control, 1.1 ± 0.1 ms; $cpx^{-/-}$, 1.8 ± 0.2 ms; OE-CPX, 1.1 ± 0.1 ms. Student's *t* test revealed significant differences between control and $cpx^{-/-}$, and OE-CPX synapses at two external Ca²⁺ concentrations. Dash lines indicate the time at which 90% of the total release has occurred during a single stimulus. *D*, Quantification of release time when 90% of the total charge was transferred in each condition. Mean release time in the strains at 0.2 mM (a^{2+} are as follows: control, 21.3 ± 2.1 ms; $cpx^{-/-}$, 37.2 ± 3.72 ms; OE-CPX, 1.5 ± 1.6 ms. Student's *t* test revealed significant differences between control and $cpx^{-/-}$ (p < 0.01), control at 0.2 and 2 mM (p < 0.01), $cpx^{-/-}$ at 0.2 and 2 mM (p < 0.01), control at 0.2 and 2 mM (p < 0.01), and OE-CPX (p < 0.01), control at 0.2 and 2 mM (p < 0.01), $cpx^{-/-}$ at 0.2 and 2 mM (p < 0.01). And DE-CPX 4.2 and 2 mM (p < 0.01), $cpx^{-/-}$ to 0.2 and 2 mM (p < 0.01). And DE-CPX 4.2 and 2 mM (p < 0.01). Control and $cpx^{-/-}$ (p < 0.01), control at 0.2 and 2 mM (p < 0.01), $cpx^{-/-}$ to 0.2 and 2 mM (p < 0.01), and OE-CPX 4.2 and 2 mM (p < 0.01). Control and $cpx^{-/-}$ (p < 0.01), control at 0.2 and 2 mM (p < 0.01), $cpx^{-/-}$ to 0.2 and 2 mM (p < 0.01). And DE-CPX 4.5 2 and 2 mM (p < 0.01). The data in all panels r

number of vesicles in the IRP can be estimated by integrating release during the depression and subtracting the constant RRP contribution. The average IRP in controls is 200 \pm 19 vesicles (Fig. 4A-D). We previously quantified active zone number at Drosophila muscle 6 in our control line (Huntwork and Littleton, 2007). Muscle 6 contains \sim 500 active zone release sites, suggesting the IRP represents ~0.4 vesicles/active zone at resting synapses (similar to what we measured for membrane docked vesicles per active zone; see Fig. 6G,H). The IRP is reduced in $cpx^{-/-}$ to 78 ± 8 vesicles (Fig. 4*C*,*D*). In contrast, CPX overexpression enhances the IRP pool size to 284 ± 28 vesicles (Fig. 4C,D). The second component of depression occurs after the initial rapid reduction in release and continues for up to the ~1000th stimuli before reaching steady-state levels (see control in Fig. 4*E*). This phase of depression represents delivery of vesicles from the RRP, and can be calculated by measuring the integral of the decaying evoked response minus the steady-state sustained component of release that is eventually reached and associated with SV recycling dynamics (Delgado et al., 2000). CPX levels had a strong impact on the RRP (Fig. 4G,H). The RRP pool size in control was 31,600 \pm 3000 vesicles, while $cpx^{-/-}$ had an RRP of 17,400 \pm 1800 vesicles (Fig. 4*G*,*H*). Animals overexpressing CPX displayed an increased RRP size of 56,700 \pm 5500 vesicles (Fig. 4G,H). By comparing the sustained response after the 1000th stimuli to continued 10 Hz stimulation in mutant and overexpression lines, we found no change in the size of the overall



Figure 4. CPX controls short-term depression and the size of the IRP and RRP vesicle pools. *A*, Representative EPSCs in control (black), $cpx^{-/-}$ (red), and CPX overexpression (OE CPX, blue) synapses during a short 10 Hz tetanic stimulation of the nerve in 2 mm external Ca²⁺. *B*, The average for the first 10 responses normalized to the first response during the tetanus in control (black), $cpx^{-/-}$ (red), and CPX overexpression (blue) synapses. *C*, Average quantal content released during 20 responses in each genotype. The IRP in each genotype was estimated during the first depression phase by taking the integral of release and subtracting the semi steady-state (shown in the inset). *D*, Quantification of the IRP vesicle pool size for each genotype in quanta are as follows: control, 200 ± 19; $cpx^{-/-}$, 78 ± 8; 0E CPX, 284 ± 28. Student's *t* test revealed significant differences between control and $cpx^{-/-}$ (p < 0.01) and control and 0E-CPX (p < 0.01) *E*, Representative EPSCs in control, $cpx^{-/-}$, and CPX overexpression synapses during a long tetanic 10 Hz nerve stimulation. *F*, The average for 1500 responses normalized to the first response during the tetanus is shown for control (black), $cpx^{-/-}$ (red), and CPX overexpression (blue) synapses. *G*, Average quantal content released during the tetanus is shown for each genotype. The average quantal content released at steady state at the end of the stimulation in the genotypes are as follows: control, 106 ± 10 quanta; $cpx^{-/-}$, 98 ± 9 quanta; OE-CPX, 110 ± 12 quanta. The integral minus the steady-state response provides an estimate of the RRP and is shown in the inset. *H*, Quantification of the average RRP in each genotype. Average values in the genotypes in kilo quanta are as follows: control, 31.6 ± 3.0 ; $cpx^{-/-}$, 17.4 ± 1.8 ; OE-CPX, 56.7 ± 5.5 . Student's *t* test revealed significant differences between control and $cpx^{-/-}$ (p < 0.01) and control and OE-CPX (p < 0.01). All recording were performed at 2 m

recycling rate (Fig. 4*E*). The average quantal content during steady-state release is 106 ± 10 quanta/stimulus in controls, compared with 98 \pm 9 quanta/stimulus in $cpx^{-/-}$ and 110 ± 12 quanta/stimulus in overexpression lines.

To further analyze use-dependent synaptic properties modulated by CPX, release during high-frequency stimulation in lower Ca²⁺ (0.2 mM) was assayed. Control synapses rapidly facilitate in low Ca²⁺, followed by a slower augmentation of the response (Fig. 5*F*). The increased release in low Ca^{2+} is hypothesized to be due to enhanced fusion from the IRP and potentiation of RRP exocytosis during the stimulus train. As shown in Figure 5A-D, $cpx^{-/-}$ synapses failed to show a rapid facilitation, while CPX overexpression resulted in faster and more robust facilitation than control synapses. Similarly, when we assayed EPSC responses during the later period of tetanic stimulation in low Ca²⁺, we observed that while control synapses potentiated their response twofold by the 500th stimuli, $cpx^{-/-}$ mutants showed a reduced augmentation of only 1.3-fold (Fig. 5F-I). CPX overexpression increased EPSC amplitude threefold by the 500th stimuli, resulting in an enhanced augmentation. Quantification of the total vesicle number released during the stimulation protocol is shown in Figure 5J. Together, these findings indicate CPX not only regulates evoked release kinetics, but also controls the size of the IRP and RRP-key elements that define the ability of synapses to sustain responses during burst firing.

One mechanism by which CPX might control the size of the releasable vesicle pool is through its ability to regulate spontaneous release rates. The dramatic enhancement in spontaneous fusion in $cpx^{-/-}$ could partially deplete vesicles within the IRP and RRP pools, leading to alterations in vesicle dynamics during tetanic stimulation. To determine whether this model would be predictive for the depletion in vesicle pools we observe, we first analyzed how increased spontaneous release would deplete vesicle number. $cpx^{-/-}$ mutant synapses show a stable resting potential baseline despite the elevated mini frequency (Fig. 6A). This allowed us to determine the cumulative spontaneous release by integrating charge transferred during rest at $cpx^{-/-}$ synapses (Fig. 6*B*). Using this method of quantification, $cpx^{-/-}$ mutants release between 150 and 200 vesicles per second through spontaneous fusion (Fig. 6C). A similar rate of vesicle fusion can be triggered at control synapses by stimulating at 0.5 Hz in 2.0 mM Ca^{2+} (Fig. 6D). This stimulation causes a steady-state release of \sim 200 vesicles per stimulus by the 20th pulse (Fig. 6*E*), similar to the rate of quanta released at rest in $cpx^{-/-}$. Delivering a 10 Hz pulse to control synapses prestimulated at 0.5 Hz results in a severe reduction in the initial fast component of depression that corresponds to IRP pool depletion (Fig. 6D-F), similar to what we observe in $cpx^{-/-}$. These results are consistent with a loss of releasable vesicles through enhanced spontaneous release in $cpx^{-/-}$ mutants. To determine whether the defect in vesicle release during high-frequency stimulation in $cpx^{-/-}$ mutants represents functional modulation of the release machinery or defects in SV docking or SV number, we examined the ultrastructure of $cpx^{-/-}$ synapses by electron microscopy. No change in SV number at or around active zones before or after nerve stimulation was observed (Fig. 6G,H). These results indicate CPX likely me-



Figure 5. CPX regulates short-term synaptic potentiation. *A*, Representative EPSCs in low external calcium (0.2 mM) in control (black), $\varphi x^{-\prime-}$ (red), and CPX overexpression (blue) synapses during a short 10 Hz tetanic-stimulation. *B*, The average response to the first 10 stimuli normalized to the first response during the tetanus is shown for control (black), $\varphi x^{-\prime-}$ (red), and CPX overexpression (blue) synapses. The average fast tetanic facilitation in each genotype is as follows: control, 1.6 ± 0.1 ; $\varphi x^{-\prime-}$, 1.1 ± 0.1 ; 0E-CPX, 2.4 ± 0.2 . Student's *t* test revealed significant differences between control and $\varphi x^{-\prime-}$ (p < 0.01) and control and 0E-CPX (p < 0.01). The average paired-pulse facilitation at 10 Hz in independent measurements for the genotypes are as follows: control, 1.3 ± 0.1 ; $\varphi x^{-\prime-}$, 1.1 ± 0.1 ; 0E-CPX, 1.4 ± 0.1 . Student's *t* test revealed significant differences between control and $\varphi x^{-\prime-}$ (p < 0.01) and control and 0E-CPX (p < 0.01). The average values is graphed for each genotype. *E*, Quantification of the fast releasable pool in each genotype is shown. Average values in quanta are as follows: control, 265 ± 15 ; $\varphi x^{-\prime-}$, 202 ± 12 ; 0E CPX, 335 ± 18 . Student's *t* test revealed significant differences between control and $\varphi x^{-\prime-}$ (p < 0.01) and control and $\varphi x^{-\prime-}$ (p < 0.01) and control and 0E-CPX (p < 0.01). *F*, Representative EPSCs in control, $\varphi x^{-\prime-}$, 1.3 ± 0.1 ; 0E-CPX, 3.0 ± 0.2 . Student's *t* test revealed significant differences between control and $\varphi x^{-\prime-}$ (p < 0.01) and control and $\varphi x^{-\prime-}$ (p < 0.01). *B*, Representative EPSCs in control, $\varphi x^{-\prime-}$, 1.3 ± 0.1 ; 0E-CPX, 3.0 ± 0.2 . Student's *t* test revealed significant differences between control and $\varphi x^{-\prime-}$ (p < 0.01). *B*, Representative EPSCs in control, $\varphi x^{-\prime-}$, 1.3 ± 0.1 ; 0E-CPX, 3.0 ± 0.2 . Student's *t* test revealed significant differences between control and $\varphi x^{-\prime-}$ (p < 0.01) and control and 0E-CPX (p < 0.01)

diates molecular interactions required for generating fusionready synaptic vesicles (priming) that form the IRP and RRP, but does not uncouple processes required for morphological docking of vesicles at active zones.

During our high-frequency stimulation experiments, we detected an additional defect in the timing of fusion that appeared in $cpx^{-/-}$ mutants over the course of a stimulation train. $Cpx^{-/-}$ animals showed a progressive delay in the onset of the evoked response during a 10 Hz stimulation protocol. To characterize this defect, we quantified the initial rising phase of evoked responses during the first 20 stimulation events compared with the final 50 events during a 150 s 10 Hz tetanic stimulation train (Fig. 61). At control synapses, we observed a small increase in the synaptic delay of ~ 0.2 ms when comparing the initial and final set of event delay intervals. In contrast, $cpx^{-/-}$ showed a dramatic increase in synaptic delay over the course of the stimulation, increasing to ~ 1 ms by the end of the train (Fig. 61). These results indicate that loss of CPX increases synaptic delay during a stimulation train, consistent with CPX normally maintaining the rate of quantal release at control synapses.

CPX regulates SYT function during evoked and spontaneous synaptic vesicle fusion

The mechanisms that underlie the ability of CPX to clamp spontaneous release and facilitate evoked fusion are unclear. One model is that CPX directly binds the SNARE complex and clamps fusion by preventing full SNARE zippering (Giraudo et al., 2009) or generating a "zigzag" SNARE array incompatible with fusion (Kümmel et al., 2011). Consistent with a direct effect on SNARE complexes, mutations in CPX that block SNARE binding fail to rescue the increased frequency of spontaneous release (Cho et al., 2010). An additional mode of regulation suggested by our findings that CPX regulates the synchronicity and Ca²⁺ cooperativity of release is that CPX may prevent premature activation of SNARE complexes by the synchronous Ca²⁺ sensor SYT. To test this model, we generated double mutants lacking both CPX and SYT. Surprisingly, the increased spontaneous release observed in $cpx^{-/-}$ alone is completely suppressed in $syt^{-/-}$; $cpx^{-/-}$ double mutants (Fig. 7A,B). Compared with the 85.6 \pm 9.2 Hz rate of spontaneous fusion in $cpx^{-/-}$ mutants alone, $syt^{-/-}$ single mutants displayed a 6.5 \pm 1.3 Hz mini frequency and syt^{-/-}; $cpx^{-/-}$ double mutants had a rate of 6.5 \pm 0.7 Hz, indicating elevated spontaneous fusion rates in $cpx^{-/-}$ requires SYT function. Given observations that SNARE binding by SYT triggers a conformational change in the protein that mimics Ca²⁺ binding (Choi et al., 2010), CPX may clamp spontaneous release by regulating access of SYT to the SNARE complex. In the absence of CPX, SYT may prematurely engage SNAREs, adopting a normally Ca²⁺-triggered fusogenic conformation that activates the enhanced spontaneous release observed in $cpx^{-/-}$.

To determine whether CPX and SYT are also linked in their roles in triggering Ca²⁺-dependent fusion, we assayed evoked release properties in double mutant animals. Quantal content is reduced over a range of Ca²⁺ concentrations in both mutants, with a shift in co-operativity to the right in both $syt^{-/-}$ and $syt^{-/-}$; $cpx^{-/-}$ (Fig. 7*C*). In the absence of SYT alone, residual



Figure 6. Low-frequency nerve stimulation partially mimics the $cpx^{-/-}$ effect on synaptic vesicle pool size. *A*, Representative trace of spontaneous activity in $cpx^{-/-}$ synapses shows a stable baseline. *B*, Integration of the charge transferred during spontaneous activity is shown for $cpx^{-/-}$ synapses. Actual traces are shown on the left, with the integrated charge transfer shown on the right (black). *C*, Cumulative charge transferred during spontaneous activity at $cpx^{-/-}$ synapses is shown. The data reveal a linear rise in charge transfer that allows estimates of the average rate of release (red trace). *D*, Representative recording of nerve evoked EPSCs in control synapses during 10 Hz tetanic stimulation with (magenta) or without (black) a prior low-frequency stimulation to mimic vesicle depletion observed at $cpx^{-/-}$ synapses. *E*, The average quantal content is quantified for stimulation with (magenta) and without (black) a 0.5 Hz prestimulation at control synapses. *F*, The responses normalized to the first tetanic response are graphed for stimulation with (magenta) and without (black) a 0.5 Hz prestimulation at control synapses. *G*, Electron micrographs of type 1b bouton active zones from $cpx^{-/-}$ and control are shown at rest and following a 10 Hz stimulation. *H*, Quantification of SV number per T-bar active zone is plotted on the right for each condition. Scale bar, 200 nm. *I*, Rise time for EPSCs normalized for the maximum for control and $cpx^{-/-}$ during the first 20 responses compared with the last 50 responses (1450–1500) of a 10 Hz 1500 pulse stimulation train in 2 mx Ga²⁺. Averages half-time change in the rise phase of an evoked event in the genotypes are as follows: control, 0.3 ± 0.1 ms; $cpx^{-/-}$, 1.1 ± 0.1 ms. Student's *t* test revealed significant differences between control and $cpx^{-/-}$ (p < 0.001). The data represent recordings of mean responses in at least six different animals for each genotype for all panels. Error bars, SEM.

evoked SV fusion becomes asynchronous (Fig. 7D-I). Double mutants lacking both SYT and CPX reduce release more profoundly and show even slower asynchrony (Fig. 7D–I). Rise time and overall release rate are also slowed in $syt^{-/-}$; $cpx^{-/-}$ compared with either mutant alone (Fig. 7G-I). Compared with mean rise time for evoked release in control animals of 1.0 \pm 0.1 ms, syt^{-/-}; $cpx^{-/-}$ double mutants had a rise time of 5.9 \pm 0.6 ms. Single mutant analysis suggests SYT exerts a more prominent effect than CPX (mean rise time of 1.7 \pm 0.2 ms in $cpx^{-/-}$ vs 2.6 \pm 0.3 ms in syt^{-/-}), but the loss of both proteins results in additive defects in total release and the speed of quantal release (Fig. 7C–I). Mean release time in control is 8.1 ± 0.8 ms compared with 18.5 \pm 0.2 ms in $cpx^{-/-}$, 38.9 \pm 4.1 ms in $syt^{-/-}$, and 57.8 \pm 6.1 ms in $syt^{-/-}$; $cpx^{-/-}$ double mutants. These results indicate that although SYT and CPX share similarities in their ability to control evoked fusion, each protein can also contribute independently to regulate release properties.

To further probe how SYT and CPX regulate release, we assayed the effects of overexpression of SYT alone versus overexpression of both proteins together at the synapse. Overexpression of SYT triggered an enhancement of release rate and the total amount of vesicles released (Fig. 8A). SYT overexpression also prolonged the rise time from 1.1 \pm 0.1 ms in controls to 3.8 \pm 0.4 ms, indicating increased SYT levels lengthened the release window rather than simply enhancing synchronous fusion (Fig. 8*B*). We hypothesized that this effect might be mediated by a mismatch between SYT and CPX levels in regulating SNAREdependent fusion. To test this model, we co-overexpressed both SYT and CPX together. Overexpression of both proteins reduced the rise time compared with SYT alone back to near control levels (1.1 \pm 0.1 ms in controls compared with 1.8 \pm 0.2 ms in strains overexpressing both proteins, Fig. 8 B, C), indicating that increasing CPX levels can synchronize SYT function in triggering SV fusion.



Figure 7. CPX and SYT interactions during spontaneous and evoked synaptic output. *A*, Postsynaptic current recordings of spontaneous release at control (black), $\varphi x^{-/-}$ (red), $syt^{-/-}$ (aqua), and $syt^{-/-}$; $\varphi x^{-/-}$ double null (gray) synapses. *B*, Quantification of average mini frequency in each genotype. Mean mini frequency in the genotypes are as follows: control, 2.8 ± 0.3 Hz; $cpx^{-/-}$, 85.6 ± 9.2 Hz; $syt^{-/-}$, 6.5 ± 1.3 Hz; $syt^{-/-}$; $epx^{-/-}$, 6.5 ± 0.7 Hz. Student's *t* test revealed significant differences between control and $cpx^{-/-}$ (p < 0.001), control and $syt^{-/-}$; $epx^{-/-}$ (p < 0.001). *C*, Ca^{2+} co-operativity of release is shown on a double logarithmic plot for control (black), $cpx^{-/-}$ (aqua), and $syt^{-/-}$; $epx^{-/-}$ (aqua), and $syt^{-/-}$; $epx^{-/-}$ (arga). The data in *A*-*C* represent recordings of mean responses in at least four different animals for each genotype. *D*, Average macroscopic EPSCs in 2 mw external Ca^{2+} in control, $\varphi x^{-/-}$, $syt^{-/-}$, and $syt^{-/-}$; $epx^{-/-}$ synapses. Note difference in *y*-axis amplitude scale bar for the last two genotypes. Individual traces are shown below for $syt^{-/-}$ and $syt^{-/-}$; $epx^{-/-}$. Average macroscopic EPSCs normalized for the maximum amplitude is presented in a magnified time scale on the right. *E*, Average normalized responses for each genotype plotted on a semi logarithmic graph to display their components. *F*, Average normalized cumulative charge transferred in each genotype is shown. *G*, Average normalized responses for each genotype size as follows: control, 270 ± 25 nA; $epx^{-/-}$, 95 ± 10 nA; $syt^{-/-}$, 32 ± 0 . An; $syt^{-/-}$, 2.5 ± 0 . AnA. Student's test revealed significant differences between control and $epx^{-/-}$ (p < 0.001), control and $syt^{-/-}$ (p < 0.001), $epx^{-/-}$ and $syt^{-/-}$, 2.5 ± 0.4 nA. Student's test revealed significant differences between control and $epx^{-/-}$ (p < 0.001), $epx^{-/-}$ and $syt^{-/-}$,

Discussion

The regulation of SNARE activity is critical for controlling the speed and rate of synaptic vesicle fusion. Here we show that the SNARE-binding protein CPX can bidirectionally regulate spontaneous release, the speed of neurotransmission, Ca^{2+} cooperativity, and short-term synaptic plasticity. We find that the role of CPX as a fusion clamp to prevent spontaneous release results from regulation of the activity of the Ca²⁺ sensor SYT *in vivo*. CPX can also synchronize SYT activity during evoked release, suggesting a critical role for CPX as a regulator of SYT function during both spontaneous and evoked synaptic vesicle

fusion. Beyond effects on spontaneous and evoked release, loss of CPX reduced the IRP and RRP pool of vesicles, similar to observations made in *C. elegans cpx* mutants (Hobson et al., 2011). We also observed that overexpression of CPX increased both the IRP and RRP synaptic vesicle pools. These effects of CPX on vesicle pools sizes resulted in changes in multiple aspects of synaptic plasticity, including alterations in depression, facilitation, and augmentation.

Alterations in the levels of either SYT or CPX modulate whether synaptic vesicle fusion occurs spontaneously or synchronously following an action potential. Loss of CPX increases spon-



Figure 8. CPX synchronizes SYT activity to speed evoked release. *A*, Average EPSCs recorded in control (black), CPX overexpression (OE CPX, blue), SYT overexpression (OE SYT, gray), or co-overexpression of both proteins (COE SYT CPX, aqua). Mean peak amplitude in the genotypes are as follows: control, 250 ± 20 nA; OE-CPX, 315 ± 30 nA; OE-SYT, 340 ± 32 nA; COE SYT CPX, 360 ± 37 nA. Student's *t* test revealed significant differences between control and OE-CPX (p < 0.01), control and OE-SYT (p < 0.01), and OE-SYT and COE SYT CPX, 1.1 ± 0.1 ms; OE-CPX, 1.1 ± 0.1 ms; OE-SYT, 3.8 ± 0.4 ms; COE SYT CPX, 1.8 ± 0.2 ms. Student's *t* test revealed significant differences between control and OE-CPX (p < 0.01), control and OE-SYT (p < 0.01), and OE-SYT and COE SYT CPX (p < 0.01). *C*, Average normalized cumulative charge transferred in each genotype is shown. Mean time for 90% of total release in each genotype is as follows: control, 8.6 ± 0.9 ms; OE-CPX, 6.3 ± 0.6 ms; OE-SYT, 15.6 ± 1 ms; COE SYT CPX, $1.3.9 \pm 1.4$ ms. Student's *t* test revealed significant differences between control and OE-CPX (p < 0.01), control and OE-SYT (p < 0.01), and OE-SYT and COE SYT CPX, 6.3 ± 0.6 ms; OE-SYT, 15.6 ± 1 ms; COE SYT CPX, $1.3.9 \pm 1.4$ ms. Student's *t* test revealed significant differences between control and OE-CPX (p < 0.01), control and OE-SYT (p < 0.01), and OE-SYT and COE SYT CPX (p < 0.01). All experiments were performed in 2 mm external Ca²⁺. The data in all panels represent recordings of mean responses in at least five different animals for each genotype. Error bars, SEM.

taneous release rates by >40-fold, in contrast to the smaller increase in spontaneous release rate (~3-fold) observed in the absence of SYT. As such, CPX plays the major role as the synaptic vesicle fusion clamp at Drosophila synapses. Several models have been proposed to account for the ability of CPX to function as a fusion clamp. Prior studies in mammals suggested that the CPX N-terminal α -helical accessory domain that flanks the SNAREbinding region inhibits fusion by partially incorporating into the 4-helix bundle of the SNARE complex to prevent full zippering (Giraudo et al., 2009; Maximov et al., 2009). A second model suggests that CPX clamps release by cross-linking intermolecular SNARE complexes into a zig-zag array (Kümmel et al., 2011; Li et al., 2011). These in vitro assays suggest the clamping abilities of CPX may be independent of SYT. Although we previously found that SNARE binding is required for the clamping function of CPX in *Drosophila* (Cho et al., 2010), our *in vivo* analysis using syt^{-/-}; $cpx^{-/-}$ double mutants demonstrate that the increased spontaneous release in $cpx^{-/-}$ requires SYT function. Our genetic studies support a model in which CPX-bound SNAREs interact with SYT in a distinct fashion compared with when CPX is absent. Recent FRET and crystallography analysis of the SYT-SNARE complex revealed that SNARE binding by SYT triggers a conformational state of the protein that mimics Ca^{2+} binding (Choi et al., 2010; Vrljic et al., 2010). In the absence of Ca^{2+} or SNARE binding, SYT exists in an open state where the Ca^{2+} binding loops in C2A and C2B are spread apart. Upon Ca²⁺ or SNARE binding, the Ca²⁺ binding loops are brought closer together, mimicking the spacing of the membrane penetrating loops of

viral fusion proteins. A conformational change of SYT into a more fusogenic form triggered by SNARE interactions in the absence of CPX fits well with our *in vivo* observations on the mechanism of increased spontaneous fusion in *cpx* mutants.

Does coordination of SYT and CPX activity also control evoked release? We find that altering CPX levels up or down can shift the Ca²⁺ co-operativity of release and the amount and speed of quantal vesicle release, similar to defects observed though manipulations of SYT activity (Littleton et al., 1994; Yoshihara and Littleton, 2002). In addition to the regulation of Ca^{2+} cooperativity, we find that CPX functions to synchronize SYT activity in triggering fusion, as revealed by dual overexpression of the two proteins. Our findings match in vitro fusion assay systems where CPX facilitates the ability of SYT to drive Ca²⁺-dependent fusion of liposomes or cells containing surface-exposed SNAREs (Krishnakumar et al., 2011; Kyoung et al., 2011). Besides increasing Ca²⁺ levels, CPX overexpression is one of the few known mechanisms to increase the speed of quantal synaptic vesicle fusion in vivo. We find that increasing CPX levels mimics the effects of increasing external Ca²⁺ for evoked release—increasing both the amount and speed of quantal fusion.

The molecular mechanism by which CPX couples SYT to the fusion machinery to enhance the speed of nerve-triggered release is currently unclear. Prior work on synaptic transmission demonstrated that the time between action potential invasion and Ca^{2+} entry through Ca^{2+} channels is a major contributor to synaptic delay (Katz and Miledi, 1967). In addition to Ca^{2+} channel opening, another source of delay occurs between Ca^{2+} influx

and activation of the fusion machinery (Chow et al., 1994; Sabatini and Regehr, 1996). We anticipate that CPX-SYT levels would regulate this key step, perhaps through an ability of CPX-bound SNARE complexes to serve as an efficient partner for a SYT-Ca²⁺ complex. Whether SYT and CPX can bind the SNARE complex at the same time is still unclear (Tang et al., 2006; Chicka and Chapman, 2009). Besides $syt^{-/-}$ and $cpx^{-/-}$ mutants, alterations in several other presynaptic proteins have been found to increase asynchrony during evoked release. In Drosophila, synapses lacking the active zone protein Bruchpilot show defects in coupling Ca²⁺ influx to vesicle fusion, although vesicle availability is not rate-limiting (Kittel et al., 2006). At mammalian Calyx of Held nerve terminals, the active zone protein RIM contributes to synaptic coupling by increasing Ca²⁺ channel density and synaptic vesicle docking (Han et al., 2011). A potential role for SYT and CPX in coupling Ca²⁺ channel function to fusion rate could also impact release kinetics, and we cannot rule out a key role for these proteins in this aspect of vesicle fusion. Similarly, although we did not observe any effects of CPX on the steadystate release rate, or find evidence of endocytotic defects by EM, we cannot rule out that CPX plays a more subtle role in regulating vesicle recycling as well.

Given our observations at the Drosophila NMJ, we propose a model to account for the effects of SYT and CPX activity on fusion. Generation of a fusion-ready vesicle would begin with the formation of SNARE complexes between a t-SNARE acceptor complex containing SNAP-25 and Syntaxin and the v-SNARE (synaptobrevin/VAMP). The SNARE proteins would zipper together via an energetically favorable coiled-coil interaction, bringing the lipid interface of the vesicle and plasma membrane into close proximity. After the initiation of SNARE complex assembly, several possible outcomes could then be triggered. At resting synapses, CPX would bind and stabilize the SNARE complex to generate fusion-ready primed vesicles that would enter the IRP. This CPX-clamped state would have to be overcome by a favorable SYT- Ca²⁺ interaction to initiate evoked fusion. Overexpression of CPX would enhance the probability of generating the clamped state, reducing spontaneous release and increasing vesicles residing in the fusion-ready IRP pool. Ca2+-binding to SYT following an action potential would generate a more energetically favorable SYT-SNARE complex that fully zippers to trigger lipid perturbation and fusion. In the absence of CPX, the barrier to fusion normally achieved would be reduced, allowing SYT-SNARE interactions to more easily proceed to trigger enhanced spontaneous release even in the absence of Ca²⁺. One interesting observation from our data is that although CPX overexpression enhances the speed of fusion at lower Ca^{2+} (0.2 mM), it only increases the amount of evoked fusion at higher Ca²⁺ levels. If SYT and CPX compete for SNARE complex binding, the binding affinities are likely to be controlled by intracellular Ca² concentration in the case of SYT. CPX overexpression has similar effects on spontaneous release compared with evoked release in low Ca^{2+} , suggesting a strong clamping function at low Ca^{2+} . The energy necessary to liberate clamped vesicles is likely to be significantly higher following CPX overexpression. We hypothesize that as Ca²⁺ levels increase, enhanced SYT activity would surpass the energy required to unclamp more vesicles, and thus generate a larger number of vesicle fusion events.

Our results also shed light on the role of CPX and SYT in regulating whether fusion would proceed through a synchronous versus asynchronous pathway. Vesicles that have been clamped by CPX-SNARE interactions would be available for evoked synchronous release following Ca^{2+} entry. Asynchronous release,

which normally represents a small component of overall fusion, would arise from SVs outside of the CPX-primed pool. Vesicles that bypass the CPX-primed state could also fuse spontaneously to generate the normally observed low mini release rate. In the absence of CPX, the probability of entering the primed state is reduced, resulting in higher rates of spontaneous release and a greater probability of asynchronous release during stimulation. In contrast, increased CPX levels would enhance the probability of vesicles entering the clamped state, resulting in decreased spontaneous release and the lack of asynchronous fusion observed following CPX overexpression. Given the numerous observations of altered CPX levels in many neurological and psychiatric diseases (Brose, 2008), an imbalance in the CPX/SYT ratio may functionally impact synaptic release properties in a host of brain diseases. Similarly, differential expression of SYT and CPX at synaptic subtypes within the brain may also contribute to the distinct release kinetics observed at different synapses.

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