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Complexin arrests a pool of docked vesicles for fast Ca^{2+}-dependent release

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Regulated exocytosis requires that the assembly of the basic membrane fusion machinery is temporarily arrested. Synchronized membrane fusion is then caused by a specific trigger—a local rise of the Ca^{2+} concentration. Using reconstituted giant unilamellar vesicles (GUVs), we have analysed the role of complexin and membrane-anchored synaptotagmin 1 in arresting and synchronizing fusion by lipid-mixing and cryo-electron microscopy. We find that they mediate the formation and consumption of docked small unilamellar vesicles (SUVs) via the following sequence of events: Synaptotagmin 1 mediates v-SNARE-SUV docking to t-SNARE-GUVs in a Ca^{2+}-independent manner. Complexin blocks vesicle consumption, causing accumulation of docked vesicles. Together with synaptotagmin 1, complexin synchronizes and stimulates rapid fusion of accumulated docked vesicles in response to physiological Ca^{2+} concentrations. Thus, the reconstituted assay resolves both the stimulatory and inhibitory function of complexin and mimics key aspects of synaptic vesicle fusion.

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Introduction

Neurotransmitter release is characterized by the tight spatial and temporal coupling of the membrane fusion machinery to a triggering signal. To achieve a fast response time in the sub-millisecond range, a readily releasable pool of synaptic vesicles is pre-docked at the active zone of the nerve terminal, and the fusion machinery, SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins, are partially pre-assembled in a ready-to-go high-energy state (Bruns and Jahn, 1995; Hua and Charlton, 1999). On the synaptic vesicle, the v-SNARE VAMP2 (also termed synaptobrevin) is engaged in a trans-SNARE complex (also termed SNAREpin) with its cognate t-SNARE, consisting of syntaxin 1 and SNAP-25 on the plasma membrane (Söllner *et al*, 1993; and reviewed in Jahn and Scheller, 2006; Malsam *et al*, 2008).

v-/t-SNARE complexes assemble via their heptad repeat-containing SNARE motifs into a four-helix bundle (Fasshauer *et al*, 1998; Sutton *et al*, 1998). The assembly of the four-helix bundle starts at the amino-terminal, membrane-distal end of the SNAREs and progresses towards the carboxy-terminal, membrane-proximal end in a zipper-like fashion, bringing the membranes in close contact finally resulting in membrane merger (Melia *et al*, 2002; Pobbati *et al*, 2006). In fully assembled SNARE complexes, VAMP2 and syntaxin 1 form a continuous helical bundle that starts at the SNARE motif and proceeds throughout the linker regions and the trans-membrane regions (Stein *et al*, 2009). This protein folding process provides enough energy to drive lipid bilayer merger (Weber *et al*, 1998; Hu *et al*, 2003; Li *et al*, 2007).

At the neuronal synapse and generally in regulated exocytosis, the SNARE assembly process is arrested at a distinct step and the arrest is released by a local increase of the Ca^{2+} concentration. Thus, in contrast to constitutive membrane fusion, regulated exocytosis has acquired two additional components that regulate the late fusion steps—complexins (Cpx) (also termed synaphins) and the Ca^{2+} sensors synaptotagmins (Syt) (reviewed in Sudhof, 2004; Chapman, 2008; Malsam *et al*, 2008; Martens and McMahon, 2008; Rizo and Rosenmund, 2008).

Syt1, a neuronal Syt isoform, is a type I trans-membrane protein, which localizes to synaptic vesicles. It contains two C2 domains, C2A and C2B, which can bind neuronal t-SNARE proteins in a Ca^{2+}-independent manner contributing to the docking of secretory vesicles at the plasma membrane (Reist *et al*, 1998; Loewen *et al*, 2006b; de Wit *et al*, 2009). Subsequent Ca^{2+}-dependent interactions with anionic phospholipids result in a partial membrane insertion of the C2 domains resulting in lipid bilayer deformation/tubulation and membrane fusion (Hui *et al*, 2006; Martens *et al*, 2007; Shahin *et al*, 2008; Paddock *et al*, 2011). Deletion of Syt1 abolishes synchronous release *in vivo* and increases spontaneous (asynchronous) fusion events (Geppert *et al*, 1994; Littleton *et al*, 1994; Fernandez-Chacon *et al*, 2001; Pang *et al*, 2006). *In vitro* reconstitution experiments have revealed a wealth of information about Syt1 function; yet, the exact mechanism remains unclear. Reconstitution of membrane-anchored Syt1 into VAMP2 liposomes stimulates membrane fusion in a Ca^{2+}-independent manner apparently by Ca^{2+}-independent Syt1–t-SNARE and Syt1–lipid interactions (Mahal *et al*, 2002; Loewen *et al*, 2006a; Stein *et al*, 2007; Kim *et al*, 2012). Addition of Ca^{2+} showed various effects on membrane fusion ranging from: (i) no effect, (ii) either stimulation or inhibition dependent on the incorporation of anionic phospholipids (phosphatidylserine) either in the t-SNARE liposomes or v-SNARE/Syt1 liposomes, (iii) stimulation in the presence of low Ca^{2+} concentrations, but inhibition in the presence of high Ca^{2+} concentrations,

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(iv) stimulation only by high Ca^{2+} concentrations above 2 mM, (v) stimulation in the range of tens of μM to 1 mM Ca^{2+} (Mahal *et al*, 2002; Stein *et al*, 2007; Lee *et al*, 2010; Kyoung *et al*, 2011, Wang *et al*, 2011). Furthermore, in these different assays, the fusion kinetics varied broadly and only in few cases, the function of Syt1 was tested together with its companion Cpx. Thus, attempts to mimic the functions of Syt1 in reconstituted *in-vitro* assays only showed partial aspects of the *in-vivo* situation.

Cpxs are small soluble proteins, which bind to t-SNAREs with low affinity and to fully assembled SNARE complexes with high affinity (McMahon *et al*, 1995; Chen *et al*, 2002). Two isoforms, CpxI and CpxII, are expressed in neuronal cells (Reim *et al*, 2005). Cpx has multiple domains each with distinct functions. The amino-terminus shows a stimulatory role (Xue *et al*, 2007). It is followed by an inhibitory α-helix, a central SNARE-binding and SNAREpin-stabilizing α-helix, as well as a stimulatory lipid-binding carboxy-terminus (Giraudo *et al*, 2006; Yoon *et al*, 2008; Giraudo *et al*, 2009; Malsam *et al*, 2009; Maximov *et al*, 2009; Seiler *et al*, 2009). Knockout of CpxI in mice and *Drosophila melanogaster* impairs evoked exocytosis (Reim *et al*, 2001; Huntwork and Littleton, 2007). Furthermore, deletion of Cpx results in a pronounced increase in spontaneous exocytosis in *Drosophila melanogaster* and *Caenorhabditis elegans* (Huntwork and Littleton, 2007; Hobson *et al*, 2011). Consistently, knockdown of CpxI and II by RNA interference in mouse cortical neurons results in an increase of spontaneous release events (Maximov *et al*, 2009). In contrast, the double knockout of CpxI and II in mice showed a reduction in spontaneous fusion events (Xue *et al*, 2008). Thus, both inhibitory and stimulatory functions can be assigned to Cpx *in vivo*, likely reflecting the different properties of the distinct Cpx domains (Xue *et al*, 2009). Attempts to study the concerted action of Cpx together with either soluble or membrane-anchored Syt1 in reconstituted assays showed rather slow fusion kinetics or an inhibitory effect of CpxI in the Ca^{2+}-stimulated fusion reaction (Giraudo *et al*, 2006; Schaub *et al*, 2006; Chicka *et al*, 2008). A recent microscopic assay detecting single liposome fusion events demonstrated that full-length Syt1 stimulates membrane fusion in the presence of Ca^{2+} with fast kinetics and CpxI further potentiates this stimulation. However, the inhibitory function of Cpx was not detected and Ca^{2+} concentrations in the millimolar range were required to stimulate fusion (Kyoung *et al*, 2011). Thus, the exact mechanism of how Syt1 and Cpx control regulated exocytosis is still a matter of discussion and remains to be resolved.

To obtain further insight into the basic interplay of the Ca^{2+}-regulated fusion machinery, we have employed a reconstituted assay that mimics the late events in synaptic vesicle fusion as closely as possible, but bypasses the inherent complexity of a cellular environment. We used conditions that reflect the molecular properties at the neuronal synapse taking care of issues such as membrane curvature, Syt1 and SNARE densities as well as lipid composition. To achieve this, VAMP2 and membrane-anchored Syt1 were reconstituted into small unilamellar vesicles (SUVs) at their physiological molar ratios and concentrations in the membrane (Takamori *et al*, 2006). Pre-assembled t-SNARE complexes were reconstituted into giant unilamellar vesicles (GUVs) to bypass t-SNARE complex assembly, and to mimic the flat plasma membrane, respectively. Using this assay, we found that Syt1 stimulated Ca^{2+}-independent fusion by increasing vesicle docking to target membranes. Cpx blocked membrane fusion by arresting vesicles at a docked stage. The addition of physiological Ca^{2+} concentrations caused fast and synchronized vesicle fusion and the presence of Cpx further potentiated this reaction. Deletion and point mutations in Cpx and Syt1 allowed us to assign distinct functions to distinct protein domains of the Ca^{2+}-regulated machinery.

Results

A reconstituted lipid mixing assay containing v-SNARE/Syt1-SUVs and t-SNARE-GUVs

To study the role of Cpx and Syt1 in Ca^{2+}-dependent membrane fusion, we adapted a well-established *in-vitro* assay that detects lipid mixing of liposomes containing reconstituted SNAREs. When unlabelled t-SNARE liposomes fuse with v-SNARE liposomes containing the fluorescently labelled lipid species NBD-phosphatidylethanolamine (PE) and rhodamine-PE at concentrations that result in quenching of the NBD fluorescence, lipid mixing is monitored as an increase of NBD fluorescence (Struck *et al*, 1981; Weber *et al*, 1998). t-SNARE proteins were reconstituted into GUVs to allow fusion analysis by both biochemical and optical methods. Moreover, low curvature t-SNARE-GUVs better reflect the flat presynaptic plasma membrane than SUVs. Pre-assembled full-length syntaxin 1/SNAP-25 complexes were reconstituted into GUVs at a protein-to-lipid ratio of about 1/1000. VAMP2 and membrane-anchored Syt1 were reconstituted into SUVs at protein-to-lipid ratios of about 1/200 and 1/800, respectively, which corresponds approximately to the VAMP2/Syt1 protein-to-lipid ratios found in purified synaptic vesicles (Takamori *et al*, 2006).

v-SNARE- or v-SNARE/Syt1-SUVs (2.5 nmol lipid, 12.5 pmol VAMP2, 3.1 pmol Syt1) and syntaxin 1/SNAP-25-GUVs (14 nmol lipid, 14 pmol t-SNARE) were mixed in the absence or the presence of 6 μM CpxII. The six-fold molar excess of GUV lipids over SUV lipids and the large diameter of the GUVs ensures that each fusion event of an SUV with a GUV results in efficient fluorescence dequenching both at the beginning and at the end of the incubation period. To block unspecific protein–lipid interactions in the absence and presence of Ca^{2+}, Mg^{2+} was added to the samples at a final concentration of 0.5 mM (corresponding to the free concentration of Mg^{2+} in cells and representing a three-fold molar excess over lipids in the reaction). Fusion was measured for 5 min at 37°C, followed by the addition of Ca^{2+} at a final concentration of 100 μM. To control for unspecific fusion, v-SNARE/Syt1-SUVs were pre-incubated with Botulinum neurotoxin D (BoNT/D) to remove VAMP2 from the liposome surface. These control samples did not show any increase in NBD fluorescence when added to t-SNARE-GUVs, confirming that fusion was SNARE dependent. The background signal derived from these BoNT/D-treated control reactions was subtracted from the other measurements as described previously (Malsam *et al*, 2009).

In the presence of CpxII, v-SNARE/Syt1-SUVs and t-SNARE-GUVs confer fast and Ca^{2+}-synchronized lipid mixing

In agreement with other studies, membrane-anchored Syt1 significantly stimulated liposome lipid mixing in a Ca^{2+}-

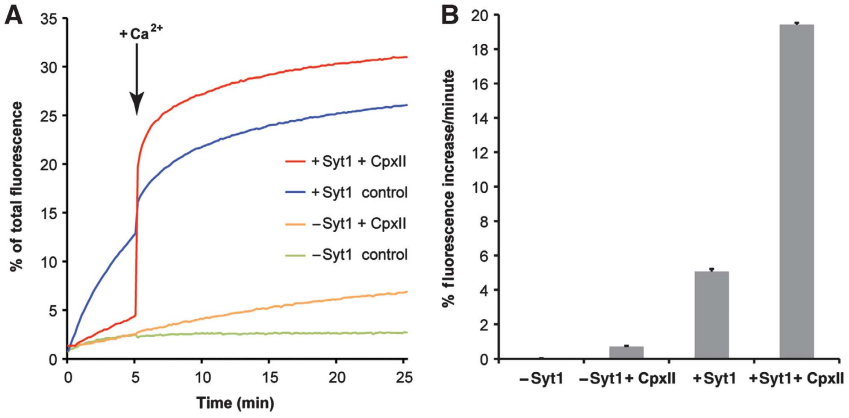

Figure 1 Fast Ca^{2+}-dependent liposome fusion requires the combined function of CpxII and Syt1. (**A**) Effect of Syt1 and CpxII on liposome fusion kinetics. v-SNARE or v-SNARE/Syt1-SUVs (2.5 nmol lipid, 12.5 pmol VAMP2, 3.1 pmol Syt1), labelled with rhodamine and NBD lipids were mixed with unlabelled t-SNARE-GUVs (14 nmol lipid, 14 pmol syntaxin 1/SNAP-25) in the absence or presence of 600 pmol CpxII in a final volume of 100 μl and the increase in NBD fluorescence was monitored. After 5 min at 37°C, Ca^{2+} was added to a final concentration of 100 μM and the measurement continued for another 20 min. The results were normalized to the maximum NBD fluorescence signal after detergent lysis as described in Materials and methods. (**B**) Comparison of the initial Ca^{2+}-dependent fusion stimulation in the presence and absence of Syt1 and CpxII. The % increase of the fluorescent signal was determined for each reaction by subtracting the value before the addition of the Ca^{2+} trigger (minute 5) from the value reached 1 min later. Error bars indicate s.e.m. ($n=3$).

independent manner (Figure 1A, blue line, first 5 min; Mahal *et al*, 2002; Stein *et al*, 2007; Kim *et al*, 2012). By comparison, the lipid mixing rate of v-SNARE liposomes lacking Syt1 was low, but could be moderately stimulated by CpxII, consistent with previous works (Figure 1A, green and orange lines) (Yoon *et al*, 2008; Malsam *et al*, 2009). Remarkably, the Ca^{2+}-independent stimulation of lipid mixing by Syt1 could be largely suppressed by the presence of CpxII (Figure 1A, first 5 min, red line). This CpxII-dependent inhibition could be converted into a rapid stimulation of lipid mixing by a Ca^{2+} trigger applied 5 min after mixing SUVs and GUVs. In the absence of CpxII, Ca^{2+}-triggered lipid mixing is also observed but less pronounced. Remarkably, in the presence of CpxII, the Ca^{2+}-dependent stimulation of lipid mixing exceeded the end signal of the CpxII-lacking control reaction, demonstrating that this assay can resolve both the inhibitory and the stimulatory function of CpxII. Statistical analyses show that the results are highly reproducible (see Supplementary Figure 1). To compare individual reactions, we quantified the change in fluorescence 1 min after the addition of Ca^{2+} (Figure 1B). Inhibition of lipid mixing by CpxII was dose dependent and reached saturation at a concentration of 2 μM (Supplementary Figure 2).

CpxII inhibits membrane fusion in the absence of Ca^{2+} by arresting vesicles at the docking stage

In order to analyse the nature of the inhibitory effect of CpxII and to determine if docked SUVs, in response to Ca^{2+}, undergo full fusion, we conducted cryo-electron microscopy (cryoEM). First, we visualized (at high magnification) the 'SUV-GUV docking phenotype' in the absence of fusion (at low temperature). t-SNARE-GUVs were mixed with CpxII and v-SNARE/Syt1-SUVs and pre-incubated on ice for 1 h before plunge freezing. The apparent size difference between t-SNARE-GUVs and docked v-SNARE/Syt1-SUVs unambiguously allowed us to distinguish both liposome populations (Figure 2A). By comparison, GUV preparations were essentially devoid of contaminating SUVs (see Supplementary Figure 3A). Second, we investigated the effect of CpxII on fusion at elevated temperature under conditions identical to those used in the lipid mixing assay. SUVs were incubated with GUVs for 5 min at 37°C in the absence or presence of CpxII, and were then imaged by cryoEM. A relative increase or decrease in fusion depending on the absence or presence of CpxII can be quantified by comparing the total numbers of SUVs present on cryoEM grids. Based on our results from the plate reader assay, we expected that in the absence of CpxII, a distinct fraction of the SUVs should be consumed by fusion with the GUVs. Consistent with this expectation, the total number of SUVs (free and docked) on the grids was 20% lower in the absence of CpxII than in the presence of CpxII (Figure 2B). Third, we analysed the number of SUVs that are docked to GUVs in these samples. The number of SUVs bound to GUVs was increased by a factor 2.2 in the presence of CpxII, indicating that CpxII promotes the accumulation of docked v-SNARE/Syt1-SUVs on the surface of t-SNARE-GUVs (Figure 2C). Representative cryoEM pictures of this docking phenotype in the absence and presence of CpxII are shown in Supplementary Figure 3B and C (at low magnification). Both, free and docked SUVs had a similar size distribution (mean diameters 62.6 ± 15.6 and 62.4 ± 14.5 nm; Supplementary Figure 4). A control reaction, in which t-SNARE-GUVs were pre-incubated with v-SNARE-SUVs lacking Syt1, did not show any SUVs docked to the t-SNARE-GUV membrane.

Fourth, we determined which percentage of the v-SNARE/Syt1-SUVs, present in the reaction mixture, fuse in a CpxII- and Ca^{2+}-dependent manner with the t-SNARE-GUVs. To determine the total number of v-SNARE/Syt1-SUVs in the incubation reaction, t-SNARE-GUVs were mixed with CpxII and v-SNARE/Syt1-SUVs at 4°C and, subsequently, were subjected to plunge freezing. About 25% of the total number of SUVs present in the reaction mixture was already bound to GUVs after a 1-min incubation at low temperature (Figure 2D). Extending this incubation time to 5 min at 37°C increased the number of v-SNARE/Syt1-SUVs that were attached to t-SNARE-GUVs to >80% in the absence of Ca^{2+}. Subsequent addition of 100 μM Ca^{2+} (1 min incubation in the presence of Ca^{2+}) triggered membrane fusion.

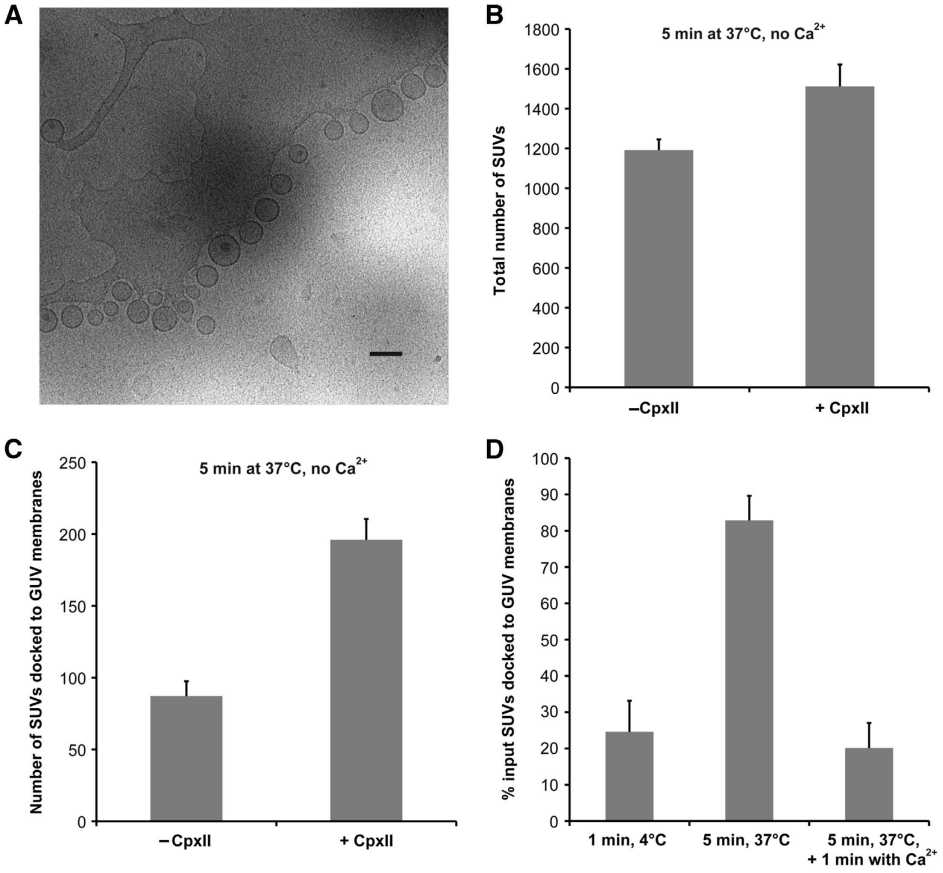

Figure 2 CpxII affects membrane fusion of v-SNARE/Syt1-SUVs with t-SNARE-GUVs. (**A**) In the presence of CpxII, v-SNARE/Syt1-SUVs accumulate on the surface of t-SNARE-GUVs. Liposomes were mixed in the presence of CpxII and incubated for 1 h on ice to maximize docking. A representative cryoEM picture shows docked v-SNARE/Syt1-SUVs. Scale bar indicates 100 nm. (**B**) CpxII blocks membrane fusion of v-SNARE/Syt1-SUVs with t-SNARE-GUVs. t-SNARE-GUVs were incubated with v-SNARE-Syt1-SUVs in the absence or presence of CpxII for 5 min at 37°C, and analysed by cryoEM. Total numbers of SUVs visible in five different images collected from three different cryoEM grids were counted. Statistics were performed 'blind'. (**C**) Accumulation of v-SNARE/Syt1-SUVs on the surface of t-SNARE-GUVs caused by the presence of CpxII. The bar diagram shows the quantification of the numbers of SUVs docked to the surface of GUVs in the absence or presence of CpxII subsequent to the 5-min incubation at 37°C (five different images collected from three different cryoEM grids). (**D**) Ca^{2+} triggers efficient fusion of the docked v-SNARE/Syt1-SUVs. Liposomes were incubated in the presence of CpxII under the following conditions and subsequently plunge frozen: (i) 1 min at 4°C; (ii) 5 min at 37°C; (iii) 5 min at 37°C, followed by the addition of 100 μM Ca^{2+} for 1 min at 37°C. The number of SUVs docked to GUV membranes in each sample was counted and the data were normalized to the total number of SUVs present in SUV-GUV incubations under non-fusogenic conditions (1 min at 4°C). Error bars indicate s.e.m. ($n = 5$).

Quantification revealed that the number of docked v-SNARE/Syt1-SUVs visible on GUV membranes was reduced by 75%, indicating that Ca^{2+} had triggered and synchronized full fusion of the majority of the docked SUVs.

The inhibitory function but not the stimulatory function of CpxI requires its accessory α-helix

In vivo and *in vitro* studies have revealed that the accessory α-helix of CpxI located in the region of amino acids 29–48 is inhibitory (Xue *et al*, 2007; Giraudo *et al*, 2008; Maximov *et al*, 2009). Therefore, we tested in the SUV/GUV fusion assay a truncation of the CpxI isoform (CpxI aa 41–134), which was used in a previous study (Xue *et al*, 2007). This CpxI construct failed to inhibit lipid mixing in the absence of Ca^{2+}, displaying kinetics identical to the control reaction lacking CpxI (Figure 3). Interestingly, the Ca^{2+}-dependent stimulation of lipid mixing of truncated CpxI still exceeded the control reaction clearly revealing a stimulatory function, which is independent of the inhibitory function.

Fast, synchronized lipid mixing triggered by physiological Ca^{2+} concentrations requires an intact Ca^{2+}-binding site in the C2B domain of Syt1

Conflicting results have been reported about the Ca^{2+} concentrations required to trigger the *in vitro* fusion of liposomes containing membrane-anchored Syt1 (Lee *et al*, 2010; Kyoung *et al*, 2011). Therefore, we analysed the concentration range of Ca^{2+} required to trigger fast Syt1- and CpxII-dependent lipid mixing by a titration experiment with EGTA-buffered Ca^{2+}. Free Ca^{2+} concentrations were monitored with a Ca^{2+}-sensitive electrode. In the presence of CpxII, Ca^{2+} triggered rapid, Syt1-dependent lipid mixing from 20 to 1000 μM Ca^{2+} (Figure 4A). Below 20 μM, stimulation of lipid mixing was still observed but significantly reduced.

In order to determine whether stimulation of lipid mixing by Ca^{2+} is directly dependent on an intact Ca^{2+}-binding loop in the C2B domain of Syt1, we reconstituted the Syt1 mutant D303/309N into v-SNARE liposomes (Supplementary Figure 5A; Fernandez *et al*, 2001; Cheng *et al*, 2004). The mutant orthologue when expressed in an Syt1 null

background in *Drosophila* neuromuscular junctions leads to a complete loss of synchronized neurotransmitter release and a shift in the Ca^{2+}-dose response curve (Mackler *et al*, 2002). Strikingly, while the mutant did not affect the SUV/GUV lipid mixing rate in the absence of Ca^{2+}, its ability to trigger Ca^{2+}-dependent lipid mixing was significantly reduced even at a concentration of 100 μM when compared with Syt1 wt (Figure 4B). Taken together, these data demonstrate that lipid mixing in the SUV/GUV assay requires an intact Ca^{2+}-binding loop in the C2B domain of Syt1 and can be triggered by Ca^{2+} over an entire concentration range from 2 μM up to 1 mM, covering physiologically relevant concentrations.

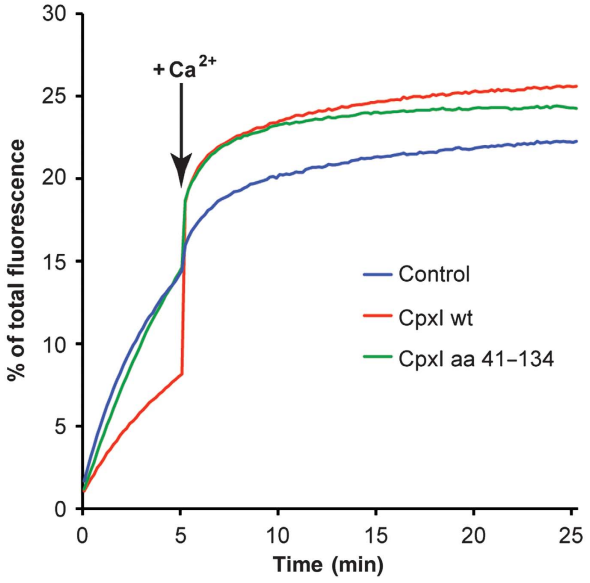

Figure 3 A CpxI construct lacking the inhibitory accessory α-helix does not inhibit Ca^{2+}-independent lipid mixing. t-SNARE-GUVs were incubated with v-SNARE-Syt1-SUVs in the absence or the presence of wild-type CpxI (CpxI wt) or a CpxI construct (CpxI aa 41–134), lacking 40 amino acids at the amino terminus. After 5 min at 37°C, Ca^{2+} was added at a final concentration 100 μM. Lipid mixing was analysed as described in Materials and methods.

The polybasic motif in the C2B domain of Syt1 is required for Ca^{2+}-independent lipid mixing

Syt1 not only stimulates liposome fusion in a Ca^{2+}-dependent manner, but also in a Ca^{2+}-independent manner (see Figure 1 and Mahal *et al*, 2002; Stein *et al*, 2007; Kim *et al*, 2012). The stimulatory function might reside in the polybasic motif in the C2B domain of Syt1, which can interact with inositol polyphosphates and the t-SNARE (Fukuda *et al*, 1995; Schiavo *et al*, 1996; Rickman *et al*, 2004). The introduction of specific mutations in the Syt1 polybasic motif completely impairs the Syt1–t-SNARE interaction *in-vitro* while *in-vivo* evoked neurotransmitter release is significantly reduced (Mackler and Reist, 2001; Loewen *et al*, 2006a). Therefore, we tested the effect of the Syt1 triple mutant K326, 327, 331Q in the SUV/GUV lipid mixing assay in the presence and absence of CpxII (Figure 5A). Indeed, v-SNARE liposomes containing the Syt1 C2B polybasic motif mutant failed to stimulate Ca^{2+}-independent lipid mixing and displayed similar kinetics to v-SNARE liposomes lacking Syt1 (compare Figure 5A, black line, 5 min, with Figure 1A; orange and green lines). The triple mutant Syt1 also largely failed to trigger rapid lipid mixing in the presence of Ca^{2+} (Figure 5, black lines, after Ca^{2+} addition).

Since the polybasic motif of Syt1 not only interacts with t-SNAREs, but also with PI(4,5)P2, we analysed lipid mixing of GUVs lacking PI(4,5)P2 (Figure 5B). Removal of PI(4,5)P2 from the t-SNARE-GUV membrane did not interfere with the Ca^{2+}-independent reaction, but selectively reduced Ca^{2+}-dependent lipid mixing. These results indicate that PI(4,5)P2 is not essential for, but clearly contributes to the Ca^{2+}-dependent fusion reaction consistent with previous studies (Stein *et al*, 2007; Lee *et al*, 2010; Kuo *et al*, 2011).

Interestingly, the ability of mutant Syt1 to trigger lipid mixing in a Ca^{2+}-dependent manner could be rescued to a significant extent by the addition of CpxII in the absence or presence of PI(4,5)P2, indicating that CpxII can compensate for the absence of the t-SNARE–Syt1 polybasic motif interaction (Figure 5, green lines).

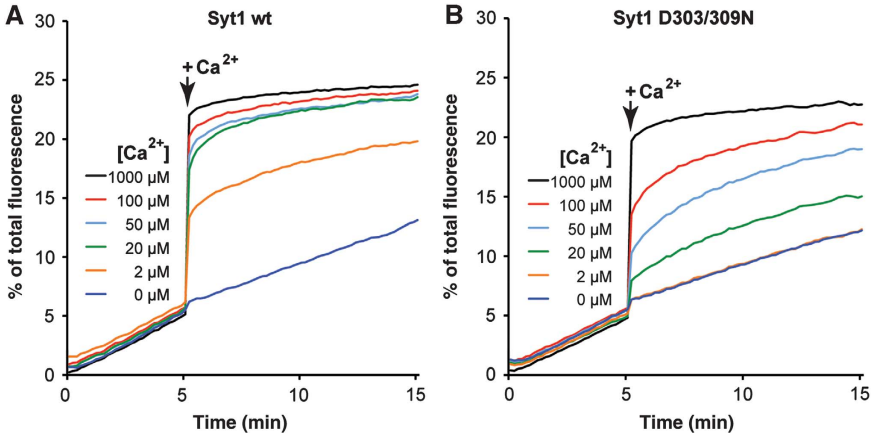

Figure 4 Ca^{2+}-dependent stimulation of lipid mixing by Syt1 requires an intact Ca^{2+}-binding site in the C2B domain of Syt1. t-SNARE-GUVs were incubated with CpxII and v-SNARE-SUVs, containing either Syt1 wt (**A**) or a Syt1 construct containing the double mutation D303/309N (**B**). After 5 min at 37°C, Ca^{2+} was added at the indicated concentrations. Lipid mixing was monitored and analysed as described in Materials and methods.

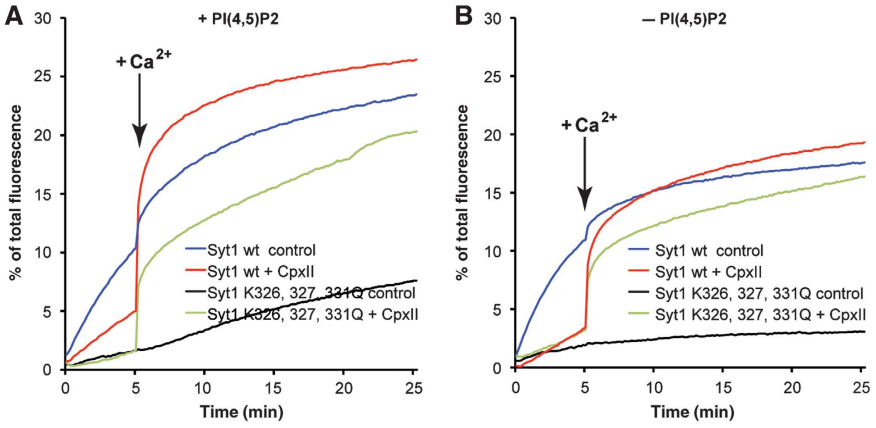

Figure 5 Ca^{2+}-independent stimulation of lipid mixing by Syt1 requires an intact polybasic motif in the C2B domain of Syt1. t-SNARE-GUVs were incubated with v-SNARE-SUVs, containing either Syt1 wt or a Syt1 construct containing the triple mutation K326, 327, 331Q in the presence or absence of CpxII. Fusion reactions were monitored in the presence (**A**) or absence (**B**) of PI(4,5)P2 and analysed as described in Materials and methods.

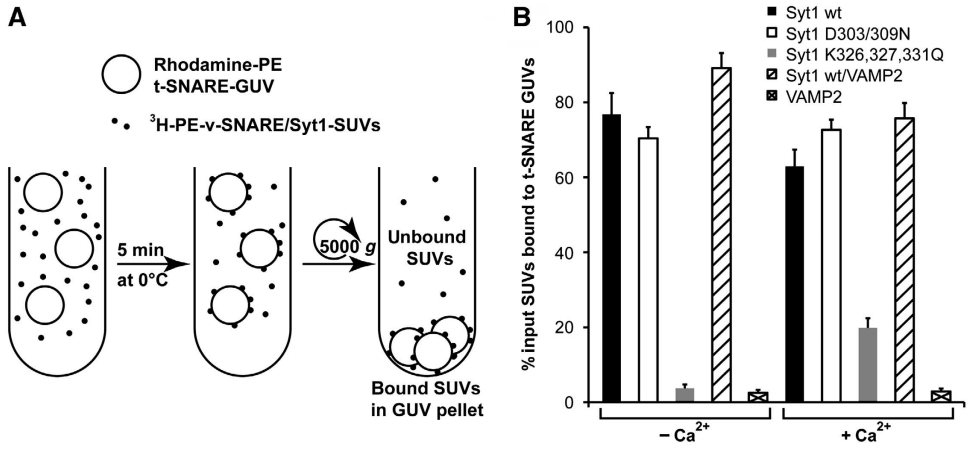

Figure 6 Docking of SUVs to t-SNARE-GUVs requires an intact polybasic motif in the C2B domain of Syt1. ^{3}H-DPPC labelled SUVs (7.5 nmol lipid) containing the indicated proteins (9.4 pmol Syt1 and/or 38 pmol VAMP2) were mixed with t-SNARE-GUVs (42 nmol lipid, 42 pmol syntaxin 1/SNAP-25) in a final volume of 200 µl in the absence or presence of 20 µM Ca^{2+} and incubated for 5 min at 4°C to allow docking. Subsequently, GUVs were isolated by centrifugation for 5 min at 5000 *g* and the fraction of bound ^{3}H-labelled SUVs in the pellet was quantified. Counts obtained from control incubations containing protein-free SUVs were subtracted from individual measurements. (**A**) Illustration of the experimental set-up. (**B**) Quantification of bound SUVs normalized to 100% SUV input. Error bars indicate s.e.m. ($n = 3$).

The polybasic motif in the C2B domain of Syt1 is required for Ca^{2+}-independent vesicle docking

The interaction of the t-SNARE with synaptotagmin 1 likely stimulates vesicle docking as has been reported in *in vitro* and *in vivo* systems (de Wit *et al*, 2009; Wang *et al*, 2011; Kim *et al*, 2012). In order to test if the Syt1 C2B polybasic motif is required for SUV-GUV docking, we established a sedimentation assay. When resuspending GUVs (filled with 250 mM sucrose) in an iso-osmolar reaction buffer of lower density, GUVs will sediment at low centrifugal force, thus allowing their separation from free SUVs, which remain in the supernatant (Figure 6A). ^{3}H-1,2-dipalmitoyl phosphatidylcholine (^{3}H-DPPC)-labelled SUVs, containing different Syt1 constructs, or VAMP2, or Syt1/VAMP2 were mixed with t-SNARE-GUVs in the absence or presence of 20 µM Ca^{2+} and were incubated for 5 min at 0°C to allow docking. Subsequently, GUVs were collected by centrifugation for 5 min at 5000 *g* and the fraction of bound ^{3}H-labelled SUVs in the pellet was quantified.

Consistent with the cryoEM studies, VAMP2 SUVs lacking Syt1 were almost completely defective in binding to GUVs in the absence or presence of Ca^{2+}, explaining their extremely slow kinetics in the lipid mixing assay (compare Figure 6B, crossed bar with Figure 1A, green and orange lines). As expected, Syt1 is the prominent docking factor and VAMP2 contributes a subtle increase of docking, which reached a maximum value of about 90% when both proteins were present on the SUVs in agreement with the cryoEM results (Figure 6B, hatched bars). In the absence of Ca^{2+}, the Syt1 C2B polybasic motif mutant was almost completely impaired in binding SUVs to t-SNARE-GUVs, demonstrating that the slow kinetics that this mutant displayed in the lipid mixing assay were due to a defect in SUV-GUV docking (compare Figure 6B, grey bars with Figure 5, black lines). In the presence of Ca^{2+}, docking could be rescued to a certain extent, consistent with Ca^{2+}-dependent Syt1/t-SNARE-lipid interactions reported in other studies (Gerona *et al*, 2000; Stein *et al*, 2007; Gaffaney *et al*, 2008). Hence, we tested the

effect of the Syt1 C2B Ca^{2+}-binding site mutant used in this study in SUV-GUV docking. As expected, Ca^{2+} did not affect the docking of this Syt1 mutant (Figure 6, white bars). In the presence of Ca^{2+}, Syt1 wt showed a small reduction in docking, which may be due to Syt1 cis-interactions with lipids located on the SUV membrane as observed by others (Stein *et al*, 2007). In summary, the data reveal that in the SUV-GUV assay presented here, docking is the rate-limiting step and requires the polybasic motif in the C2B domain of Syt1.

Soluble Syt1 constructs containing the C2B domain inhibit membrane fusion in vitro, as in vivo

In vivo, the overexpression of soluble Syt1 C2 domains in a Syt1 wt background effectively blocks evoked neurotransmitter release (Elferink *et al*, 1993; Sugita *et al*, 2002). To further validate if the reconstituted assay can reproduce observations from cellular exocytosis studies, we tested if the addition of soluble C2 domains would affect the ability of membrane-anchored Syt1 to trigger fusion in the presence or absence of Ca^{2+} and CpxII.

First, we directly compared fusion reactions containing either membrane-anchored Syt1 or the soluble C2A, C2B, C2A-B domains of Syt1. To observe stimulations with the soluble Syt1 domains, concentrations of 6 μM were used (compared with 31 nM membrane-anchored Syt1). Ca^{2+}-independent lipid mixing was not detectable when the individual C2 domains were added to a mixture of t-SNARE GUVs and v-SNARE SUVs lacking membrane-anchored Syt1, and the addition of Ca^{2+} could not synchronize the reaction kinetics, resulting only in a slow stimulation of lipid mixing (Figure 7A, orange, blue and green lines). By comparison, the linked C2A-B construct showed a weak Ca^{2+}-independent effect and a fast Ca^{2+}-dependent stimulation of lipid mixing (Figure 7A, black line). However, considering the vast concentration difference compared with membrane-anchored Syt1, the stimulation by soluble C2A-B was significantly lower.

Second, we analysed the effect of soluble C2 domains, when added to a fusion reaction containing membrane-anchored Syt1. Remarkably, addition of the soluble C2B domain to any fusion reaction (alone or in combination with the separate or linked C2A domain) almost completely abolished fusion in the absence of Ca^{2+} and resulted in significantly reduced fusion rates in the presence of Ca^{2+} (Figure 7B, black, orange and green lines). By contrast, the soluble C2A domain alone had no effect on fusion kinetics (Figure 7B, blue line). These data indicate that the addition of excess soluble C2B competes with membrane-anchored Syt1 for binding to t-SNAREs with its polybasic motif and as a result inhibits fusion. Thus, as *in vivo*, soluble domains of Syt1 suppress fusion reactions containing membrane-anchored Syt1 (Sugita *et al*, 2002).

Discussion

By using a reconstituted SUV/GUV membrane fusion assay and mimicking the cellular conditions as closely as possible, we were able to dissect the functions of Syt1 and Cpx in Ca^{2+}-regulated exocytosis.

Calcium-independent/dependent functions of Syt1 in the absence of Cpx

Membrane-anchored Syt1 stimulates the overall membrane fusion reaction in a Ca^{2+}-independent manner by increasing vesicle docking. Vesicle docking becomes the rate-limiting step in the overall fusion reaction, because low lipid concentrations (140 μM GUV lipids and 25 μM SUV lipids) were chosen. Consistently, previous *in-vitro* studies have revealed that membrane-anchored Syt1 can potentiate vesicle docking and fusion in a t-SNARE and PI(4,5)P2-dependent manner (Mahal *et al*, 2002; Bai *et al*, 2004; Loewen *et al*, 2006a; Stein *et al*, 2007; Lee *et al*, 2010; Wang *et al*, 2011; Kim *et al*, 2012). More importantly, *in vivo*, the interaction of Syt1 with syntaxin 1/SNAP-25 mediates vesicle docking (Loewen *et al*, 2006a; de Wit *et al*, 2009). Our data further substantiate that vesicle docking requires the polybasic motif of the C2B domain of Syt1. Further, in the absence of Ca^{2+} and Cpx we observe that the majority of v-SNARE/Syt1-SUVs is consumed by SNARE-mediated fusion within several minutes. The addition of Ca^{2+} to a fusion reaction,

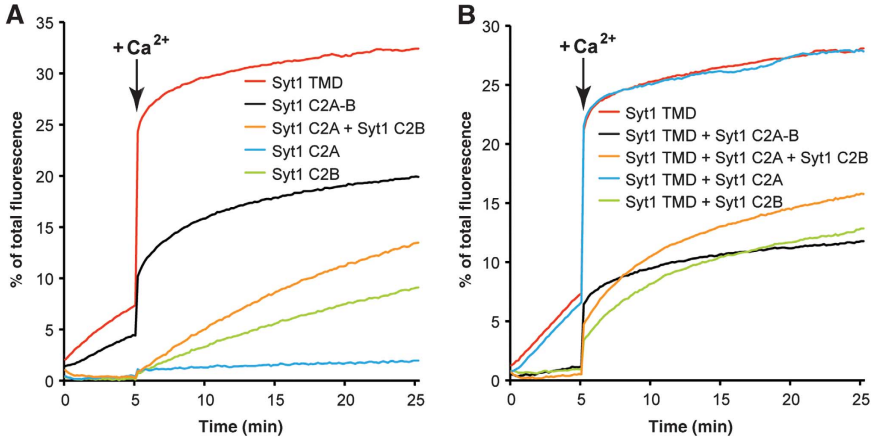

Figure 7 Effect of soluble Syt1 C2 domains on lipid mixing. (**A**) Comparison of lipid mixing reactions containing various soluble Syt1 C2 domains with a reaction containing membrane-anchored Syt1 (Syt1 TMD). t-SNARE-GUVs and v-SNARE-SUVs were incubated with CpxII in the presence of 6 μM soluble Syt1 C2A and C2B domains, which were added separately, in combination, or joined by their native linker. In the reaction, membrane-anchored Syt1 (Syt TMD) is present at 31 nM. (**B**) The soluble Syt1 C2B domain inhibits lipid mixing of v-SNARE/Syt1-SUVs with t-SNARE-GUVs. t-SNARE-GUVs and v-SNARE/Syt1-SUVs were incubated with 6 μM of the indicated Syt1 C2 constructs. After 5 min at 37°C, Ca^{2+} was added to a final concentration of 100 μM. Lipid mixing was monitored and analysed as described in Materials and methods.

which already proceeds for 5 min and lacks Cpx, triggers a rapid, albeit less pronounced increase in fusion when compared with samples containing Cpx. Apparently, in the absence of Ca^{2+}, a small but distinct pool of docked vesicles accumulates and can fuse in a synchronized manner in response to Ca^{2+}. The existence of such a vesicle pool suggests that in the absence of Ca^{2+} and in the presence of Syt1, docking does not instantly result in fusion, but likely is delayed by a clamping function of Syt1 (Chicka *et al*, 2008).

Stimulatory and inhibitory roles of Cpx

In the absence of Syt1, the SUVs and GUVs containing physiological amounts of SNAREs show very weak fusion signals. Membrane docking is rate limiting, and Cpx shows a weak stimulation, because it stimulates/stabilizes SNAREpin formation via its central α-helix (Malsam *et al*, 2009). In contrast, upon incorporation of membrane-anchored Syt1 into v-SNARE-SUVs, Syt1 significantly accelerates the docking step and now the inhibitory function of Cpx becomes prominent. Docked vesicles dramatically accumulate, because their fusion with the GUV membrane is blocked. At this stage, Cpx and Syt1 might synergize to enhance the clamping efficiency. CryoEM demonstrates an increased Cpx-dependent accumulation of v-SNARE/Syt1-SUVs on the surface of the t-SNARE-GUVs during short incubation times (5 min) at physiological temperature. Consistent with the lipid-mixing assay, the morphological analysis confirms that Ca^{2+} triggers the fusion of the docked vesicles instantly (within the technical limits of our time resolution). Subsequent to this, the fusion reaction almost ceases. These results indicate that the entire functional pool of vesicles has been consumed by full fusion in a fast reaction. Although the time resolution of the SUV/GUV fusion assay is limited, it is reasonable to speculate that the reactions are actually occurring much faster. Recent publications using a microscopic set-up to measure single vesicle fusion have revealed that SNARE mediated fusion in reconstituted assays occurs within milliseconds (Karatekin *et al*, 2010; Kyoung *et al*, 2011). Cpx inhibits fusion and accumulates docked vesicles in the *in vitro* SUV/GUV fusion assay, which suggests that *in vivo* the inactivation of Cpx would significantly increase spontaneous neurotransmitter release and simultaneously reduce the number of docked synaptic vesicles. Indeed, such a phenotype is observed in *Cpx-1* mutants in *C. elegans* (Hobson *et al*, 2011).

Remarkably, the presence of Cpx not only blocks the Ca^{2+}-independent reaction and efficiently synchronizes the fusion reaction; it also increases the pool of liposomes which fuses in a Ca^{2+}-dependent manner, consistent with a Cpx-dependent vesicle priming reaction *in vivo* (Cai *et al*, 2008; Hobson *et al*, 2011). These dual properties—inhibition and stimulation—reflect the functions of Cpx reported in the cellular environment (Yang *et al*, 2010; Hobson *et al*, 2011). At the molecular level, the binding of Cpx to partially assembled SNAREpins likely stabilizes and increases the pool of productive (primed) SNAREpins (Malsam *et al*, 2009; Li *et al*, 2011). The inhibitory/accessory α-helix of Cpx simultaneously blocks further SNAREpin assembly. Recent structural studies suggest a detailed atomic model of the Cpx–SNARE interaction and indicate how this block could function. Cpxs bridge SNARE complexes and arrange them in a zigzag array (Kummel *et al*, 2011). The central Cpx α-helix stabilizes the SNAREpin and the accessory α-helix binds the neighbouring SNAREpin, occupying the binding site for the membrane-proximal half of the VAMP2 SNARE motif, thereby blocking completion of SNAREpin zippering. Consistent with this working model, the removal of the inhibitory α-helix completely abolishes the inhibitory effect of Cpx in the SUV/GUV fusion assay, while the stimulatory function of Cpx persists (observed as an increased pool of vesicles fusing in a Ca^{2+}-dependent manner). Thus, Cpx binding stabilizes SNAREpins likely via its central α-helix domain. Nevertheless, it is also possible that, in addition, other regions of Cpx, like the carboxy-terminal domain, further control the Ca^{2+}-dependent reaction via interactions with lipids or Syt1 (Tokumaru *et al*, 2008; Yoon *et al*, 2008; Seiler *et al*, 2009).

It should also be kept in mind that the inhibitory effect of Cpx must be readily releasable, and thus a delicate balance between inhibition and release must exist. Thus, it is not surprising that a subtle tilt in this balance, for example, changes in the expression levels of Cpx, could profoundly affect regulated exocytosis (Itakura *et al*, 1999). Interestingly, CpxII expression levels are altered in some neurological disorders such as schizophrenia (Begemann *et al*, 2010).

Role of PI(4,5)P2 in synchronized vesicle fusion

Previous studies, which also employed membrane-anchored Syt1 (in the absence of Cpx), have shown that Syt1 can inhibit fusion in a Ca^{2+}-dependent manner (Stein *et al*, 2007). This type of inhibition depends on the presence of anionic lipids in the v-SNARE/Syt1-SUVs, and is caused by Ca^{2+}, which directs the binding of the C2 domains of Syt1 in *cis* to the v-SNARE/Syt1-SUVs. The SUVs and GUVs in our assay contain complex lipid compositions, which are similar on both liposomes with the exception of PI(4,5)P2. Thus, the presence of PI(4,5)P2 could steer the C2 domains of Syt1 in a Ca^{2+}-independent reaction to the GUV membrane (Bai *et al*, 2004). In a recent study, PI(4,5)P2 together with a pre-incubation step of t-SNARE SUVs and v-SNARE SUVs containing full-length Syt1 was absolutely required to synchronize lipid mixing in a Ca^{2+}-dependent manner, albeit with a moderate response (Wang *et al*, 2011). However, Cpx was lacking in these incubations. Another study, performed in the absence of Cpx, indicates that the Ca^{2+}-dependent interaction of synaptotagmin 1 with ternary SNARE complexes also requires PI(4,5)P2 (Kim *et al*, 2012). Our data show that in the presence of CpxII, fast synchronized fusion is maintained even in the absence of PI(4,5)P2, indicating a tight functional coupling of Syt1 and Cpx which is discussed below. Nevertheless, consistent with the recent work by Wang *et al* and Kim *et al*, we assume that the pre-incubation of the SUVs and GUVs in the absence of Ca^{2+} favours an initial Syt1 t-SNARE-GUV trans-interaction. This interaction is sufficient to place Syt1 in a functional position, in analogy to the physiological situation where a readily releasable pool of synaptic vesicles is docked at the active zone of the plasma membrane and subsequently responds to the Ca^{2+} trigger. Taken together, although PI(4,5)P2 is not essential to synchronize lipid mixing in the presence of Cpx, it seems to potentiate a trans-interaction of Syt1 with the GUV membrane and increases the Ca^{2+}

sensitivity of the reaction (Lee *et al*, 2010; van den Bogaart *et al*, 2012).

Ca^{2+} dependence of the v-SNARE/Syt1-SUV t-SNARE-GUV fusion reaction

Ca^{2+} concentrations above 20 μM result in efficient lipid mixing, but already low concentrations such as 2 μM Ca^{2+} showed some stimulation, which is in good agreement with the broad range of Ca^{2+} requirements reported for cellular exocytosis events (Heidelberger *et al*, 1994; Voets *et al*, 2001; Kreft *et al*, 2003; Schneggenburger and Neher, 2005). However, a straight forward comparison is hampered by the presence of various synaptotagmin isoforms in living cells. In the SUV–GUV fusion assay, we neither observe an increased inhibition at Ca^{2+} concentrations above 20 μM nor do we need Ca^{2+} concentrations of 5 mM to observe efficient fusion as reported in other liposome fusion assays (Lee *et al*, 2010; Kyoung *et al*, 2011). Thus, our data support a model where Syt1 functions as a low-affinity Ca^{2+} sensor that triggers rapid vesicle exocytosis in response to high Ca^{2+} concentrations in the range of 10–200 μM (Shin *et al*, 2002; Sudhof, 2002). Undoubtedly, Ca^{2+}-dependent fusion in the SUV/GUV fusion assay is mediated by the Ca^{2+} sensor Syt1, because mutations in the Ca^{2+}-binding site of the C2B domain significantly shift the Ca^{2+} response curve to higher Ca^{2+} concentrations. In neuromuscular junctions of *Drosophila melanogaster*, this mutant also shows a shift in the Ca^{2+} curve (Mackler *et al*, 2002). An increase of the extracellular Ca^{2+} concentrations from 2 to 5 mM Ca^{2+} is not sufficient to rescue the severe exocytosis phenotype; however, the actual local intracellular Ca^{2+} concentration is not known (Mackler *et al*, 2002).

Effect of soluble Syt1 constructs on v-SNARE/Syt1-SUV t-SNARE-GUV fusion

As in cellular exocytosis, addition of soluble Syt constructs containing the C2B domain inhibits membrane fusion (Sugita *et al*, 2002). The addition of the soluble Syt1 C2A domain to the SUV/GUV assay did not show any effect, which seems to be inconsistent with cellular studies in which the injection of the C2A domain into PC12 cells clearly inhibited exocytosis (Elferink *et al*, 1993). However, analyses of the *in-vivo* block revealed that the inhibition selectively required the polybasic motif of the C2A domain, which interacts with and modulates Ca^{2+} channel activities (Thomas and Elferink, 1998; Cohen *et al*, 2003, 2008). Since our minimal system lacks Ca^{2+} channels, the C2A domain should not inhibit membrane fusion.

Tight functional coupling of Syt1 and Cpx

Surprisingly, Cpx can rescue at least partially fast Ca^{2+}-dependent membrane fusion in the presence of the Syt1 C2B polybasic mutant. As already noted, this mutant is defective in binding inositol polyphosphate and the t-SNARE to a large degree (Fukuda *et al*, 1995; Schiavo *et al*, 1996; Rickman *et al*, 2004). Consequently, both Syt1-stimulated vesicle docking and the resulting Ca^{2+}-independent fusion stimulation are almost completely lost. In addition, a Ca^{2+}-evoked stimulation is not detectable. Remarkably, the addition of Cpx partially, but selectively rescues the Ca^{2+}-dependent membrane fusion. Since Cpx itself does not significantly stimulate the fusion reaction in a Ca^{2+}-independent or dependent manner, it must affect Syt1 function, whose Ca^{2+}-binding sites are still intact in the polybasic mutant. Cpx could rearrange or position Syt1 into a constellation favouring membrane fusion. If such a reaction requires binding of Cpx to the SNAREpin or to Syt1 remains to be shown. Although the exact molecular mechanism is unclear, our *in-vitro* data closely reflect the *in-vivo* phenotype of the polybasic Syt1 mutation in *Drosophila melanogaster*, which shows a two-fold reduction in evoked transmitter release (Mackler and Reist, 2001; Loewen *et al*, 2006a).

In summary, we have faithfully reconstituted the final steps of Ca^{2+}-triggered membrane fusion in a liposome fusion assay. The data reveal that Cpx causes the accumulation of a docked pool of vesicles, which in the presence of Syt1 instantly responds to a Ca^{2+} trigger in the physiological concentration range, resulting in rapid and synchronized membrane fusion, possibly reflecting the last moments in the lifetime of synaptic vesicles. This *in-vitro* assay also offers the opportunity to test the mechanistic role of other components known to regulate vesicle docking and priming such as Munc13 and Munc18.

Materials and methods

DNA manipulation and plasmid construction

DNA constructs were made using standard genetic manipulations. Phusion polymerase, restriction enzymes and ligases were from New England Biolabs. The *Escherichia coli* strain XL1-blue (Stratagene) was used for standard cloning. The following commercially available expression vectors were used in this work: pET15b, pET24a, pET3a (Novagen) and pGEX-4T1 (Stratagene). Hexahistidine and glutathion S-transferase tagged proteins are referred in the text as His_6 or GST proteins, respectively. The identity of all constructs was checked by DNA sequencing. DNA constructs encoding the various proteins are described in detail in Supplementary Data online.

Protein reconstitution into liposomes

All lipids were from Avanti Polar Lipids with the exception of ^{3}H-DPPC, which was from Amersham Pharmacia Biotech. v-SNARE/Syt1 lipid mix = 30 mol% POPC (1-palmitoyl-2-oleoyl-SN-glycero-3-phosphatidylcholine), 15 mol% DOPS (1,2-dioleoyl-SN-glycero-3-phosphatidylserine), 22.6 mol% POPE (1-hexadecanoyl-2-octadecenoyl-SN-glycero-3-phosphoethanolamine), 5 mol% liver PI (L-α-phosphatidylinositol), 25 mol% cholesterol (from ovine wool), 1.6 mol% Rhodamine-DPPE (*N*-(lissamine rhodamine B sulfonyl) 1,2-dipalmitoyl phosphatidylethanolamine), 0.8 mol% NBD-DPPE (*N*-(7-nitro-2,1,3-benzoxadiaziole-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine) and trace amounts of ^{3}H-DPPC (1,2-dipalmitoyl-phosphatidylcholine), 3 μmol total lipid. Syntaxin1/SNAP25 lipid mix = 35 mol% POPC, 15 mol% DOPS, 20 mol% POPE, 3 mol% liver PI (L-α-phosphatidylinositol), 2 mol% brain PI(4,5)P2 (L-α-phosphatidylinositol-4,5-bisphosphate), 25 mol% cholesterol (from ovine wool) and trace amounts of ^{3}H-DPPC, 5 μmol total lipid.

SUVs were formed in the presence of VAMP2 (protein-to-lipid ratio 1/200) and Syt1 (1/800) using the lipid mix defined above and the previously described technique of dilution and dialysis followed by a Nycodenz gradient centrifugation (Weber *et al*, 1998). The preparation of t-SNARE GUVs is described below. The protein expression and purification are described in detail in Supplementary Data. Protein amounts in the reconstituted liposomes were determined by SDS–PAGE analyses and Coomassie blue staining using BSA protein standards followed by quantification with ImageJ 1.440 (National Institutes of Health, USA) software. Lipid recoveries were determined using ^{3}H-DPPC.

Preparation of giant unilamellar t-SNARE vesicles

In all, 5 μmol dried lipids (PC:PS:PE:chol:PI:PI(4,5)P2 = 35:15:20:25:3:2) were dissolved with 5 nmol t-SNARE complex in a final volume of 0.7 ml containing 1.7% octyl-β-D-glucopyranoside. Small unilamellar t-SNARE vesicles were formed by detergent dilution with a three-fold volume of reconstitution buffer (25 mM HEPES-

KOH, pH 7.4, 400 mM KCl, 1 mM DTT). The liposome suspension was desalted using a PD10 column (GE Healthcare) equilibrated with drying buffer (1 mM HEPES-KOH, pH 7.4, 20 mM Trehalose, 1% glycerol, 1 mM DTT) and snap frozen in liquid nitrogen. In all, 1 ml thawed liposome suspension was loaded onto a 30-ml Sephadex-G50 superfine gel filtration column (GE Healthcare) equilibrated with drying buffer to remove trace contaminants of detergent and salt. In all, 3 ml eluate were collected and liposomes sedimented in a TLA-55 rotor (Beckman) at 55.000 r.p.m. for 2 h at 4°C. Pellets were resuspended in a total volume of 15 µl each and spread as uniform layer (diameter around 10 mm) on the surface of ITO-coated glass slides (GeSIM mbH). The liposome suspension was dried for 1 h at low vacuum (100 mbar), followed by another hour at high vacuum (1 mbar). An O-ring (20 × 2 mm) was used to seal the chamber and electroformation of GUVs was performed in GUV buffer (1 mM HEPES-KOH, pH 7.4, 0.25 M sucrose (Ca^{2+} free from FLUKA), 1 mM DTT) for 15 h at 10 Hz and 1 V at 0°C. GUVs were detached from the glass plate by gentle pipetting and collected at 1000 *g* for 20 min at 4°C after mixing with a 10-fold volume of iso-osmolar glucose in the same buffer. Pellets were resuspended in 90 µl fusion buffer (25 mM HEPES-KOH, pH 7.4, 135 mM KCl, 1 mM DTT) and used in subsequent fusion or binding assays.

Fusion assays

Fusion reactions and data analysis were performed as described previously (Weber *et al*, 1998) with the following modifications: (i) fusion buffer was 25 mM HEPES-KOH, pH 7.4, 135 mM KCl, 1 mM DTT (dithiothreitol); (ii) in all cases, 14 nmol (lipid) of t-SNARE-GUVs and 2.5 nmol (lipid) of v-SNARE/Syt1-SUVs were used in a final volume of 100 µl; (iii) unless otherwise noted, liposomes were mixed with 100 µM EGTA-KOH, pH 7.4 and 0.5 mM $MgCl_2$ in the presence or absence of CpxI or II with or without 6 µM soluble Syt1 constructs and quickly transferred to a pre-warmed microtiter plate. Samples were measured at 37°C in a Synergy 4 plate reader (BioTek Instruments GmbH) at intervals of 10 s. After 5 min, Ca^{2+} was added to a final concentration of 100 µM (or as noted otherwise) by the addition of 20 µl of a 6× stock solution (6.6 mM Ca^{2+}, 5.5 mM EGTA). Free Ca^{2+} concentrations were calculated by using the MAXCHELATOR software (http://maxchelator.stanford.edu; Schoenmakers *et al*, 1992). (iv) The NBD fluorescence obtained from control incubations containing v-SNARE/Syt1-SUVs pretreated with BoNT/D were subtracted from individual measurement sets. (v) The fusion-dependent fluorescence was normalized to the maximal fluorescent signal obtained in the presence of 0.4% dodecylmaltoside (Fluka). Fusion kinetics displayed in all figures show one representative experiment. Experiments were repeated several (3–4) times independently, yielding virtually identical results. For statistical analysis, changes in fusion kinetics in response to the Ca^{2+} trigger were quantified as change in % of total fluorescence 1 min after the addition of Ca^{2+} (the 1-min time window was chosen, because the addition of Ca^{2+} to the samples in the plate reader takes about 10–30 s).

CryoEM

Samples were transferred onto freshly glow discharged C-Flat™ (Protochips) holey carbon grids in a high-humidity chamber (EMBL Heidelberg). Blotting was carried out from the opposite side of the grid with a filter paper that was extensively washed previously with buffer containing 50 mM EDTA. Immediately after blotting, the samples were plunge frozen in liquid ethane and stored in liquid nitrogen. Samples were imaged under standard low-dose conditions in an FEI CM120 Biotwin microscope (120 kV) equipped with a Gatan 626 cryo-holder. Images of each sample were collected at either × 4000 or at a × 33 000 magnification, giving an unbinned pixel size of 2.5 or 0.36 nm, respectively. The total number of SUVs and the number of bound SUVs in randomly selected low-magnification images was recorded for each sample.

GUV sedimentation assay

SUVs (7.5 nmol lipid, 9.4 pmol Syt1 or 38 pmol VAMP2) labelled with ^{3}H-DPPC were mixed with GUVs (42 nmol lipid, 0.84 nmol PI(4,5)P2, 42 pmol t-SNARE) in a final volume of 200 µl in the absence or presence of 20 µM Ca^{2+} and incubated at 0°C for 5 min to allow docking. Subsequently, GUVs were sedimented at 5000 *g* for 5 min at 0°C and the fraction of bound ^{3}H-labelled SUVs in the pellet was determined. Counts obtained from control incubations containing protein-free SUVs (about 5% of the input) were subtracted from individual measurement sets. Quantitation of bound SUVs was normalized to 100% SUV input.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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