

# Rearrangements under confinement lead to increased binding energy of Synaptotagmin-1 with anionic membranes in $Mg^{2+}$ and $Ca^{2+}$

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**Synaptotagmin-1 (Syt1) is the primary calcium sensor ( $Ca^{2+}$ ) that mediates neurotransmitter release at the synapse. The tandem C2 domains (C2A and C2B) of Syt1 exhibit functionally critical,  $Ca^{2+}$ -dependent interactions with the plasma membrane. With the surface forces apparatus, we directly measure the binding energy of membrane-anchored Syt1 to an anionic membrane and find that Syt1 binds with  $\sim 6 k_B T$  in EGTA,  $\sim 10 k_B T$  in  $Mg^{2+}$  and  $\sim 18 k_B T$  in  $Ca^{2+}$ . Molecular rearrangements measured during confinement are more prevalent in  $Ca^{2+}$  and  $Mg^{2+}$  and suggest that Syt1 initially binds through C2B, then reorients the C2 domains into the preferred binding configuration. These results provide energetic and mechanistic details of the Syt1  $Ca^{2+}$ -activation process in synaptic transmission.**

**Keywords:** membrane fusion; neurotransmission; synaptotagmin

Upon arrival of an action potential at the neuronal synapse, calcium ions ( $Ca^{2+}$ ) enter the cytosol of the neuron, triggering the soluble *N*-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins to fully zipper, leading to fusion of predocked vesicles containing neurotransmitters [1,2]. Synaptotagmin-1 (Syt1), a synaptic vesicle associated protein, has been identified as the principal  $Ca^{2+}$  sensor that activates SNAREs following  $Ca^{2+}$  influx [1,3,4]. Syt1 has two  $Ca^{2+}$  binding C2 domains (C2A and C2B), connected by a 9-residue flexible linker domain, and a 61-residue linker domain between the transmembrane

domain and the tandem C2 domains. Syt1 is known to interact with the plasma membrane both before and after  $Ca^{2+}$  binding. A polylysine stretch in C2B has been shown to interact with anionic lipids in the absence of  $Ca^{2+}$  [5–8]. Upon  $Ca^{2+}$  influx, three  $Ca^{2+}$  bind in C2A and two  $Ca^{2+}$  bind in C2B.  $Ca^{2+}$  binding to the anionic pocket effectively neutralizes electrostatic repulsion between the binding site and the target membrane [9], which allows nonpolar residues nearby the  $Ca^{2+}$  binding sites (also referred to as hydrophobic loops) to insert into the membrane [9–12], serving as the power stroke to activate fusion.

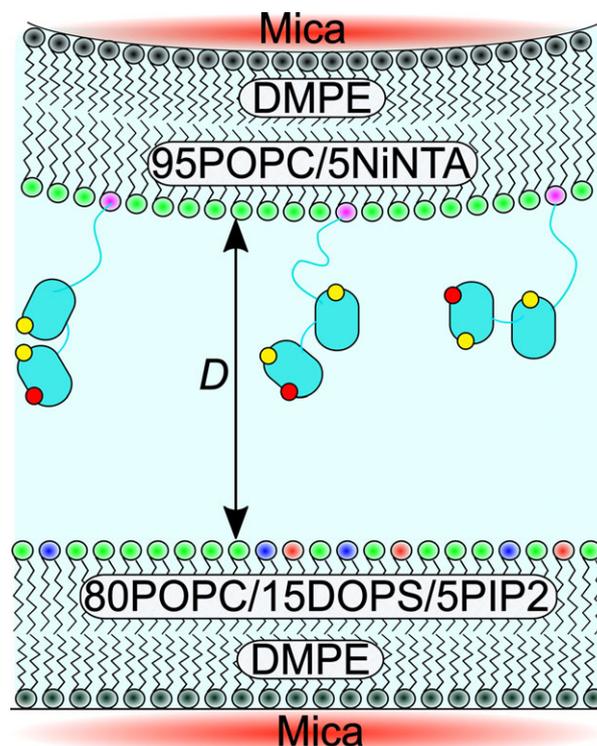
## Abbreviations

DMPE, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine; FRET, fluorescence resonance energy transfer; MST, microscale thermophoresis; PIP2, phosphatidylinositol; PS, phosphatidylserine; SFA, surface forces apparatus; Syt1, synaptotagmin-1.

The precise biochemical and biophysical mechanisms for the Syt1  $\text{Ca}^{2+}$ -trigger remain unclear. While mutating the C2A  $\text{Ca}^{2+}$  binding site causes a significant decrease to evoked neurotransmitter release *in vivo*, an analogous mutation in C2B effectively abolishes evoked release [13,14]. Several studies illustrate that Syt1 can oligomerize and modulate bending of the target membrane and that C2B drives these processes [4,15,16]. Syt1 is therefore thought to function at least partially by performing bending work on the target membrane, providing a highly curved membrane to ease the high energy barrier associated with membrane fusion. Syt1 may also act as a clamp in the absence of  $\text{Ca}^{2+}$ , by forming oligomerized structures which keep the SNAREs away from the vesicle-membrane contact zone [16–19].

The kinetics and assembly of soluble C2AB (i.e. Syt1 without the transmembrane domain and linker domain, containing only the soluble C2A and C2B domains) with anionic membranes have been assessed using stopped-flow fluorescence resonance energy transfer (FRET) and microscale thermophoresis (MST) measurements, revealing strong binding between soluble C2AB and membranes containing phosphatidylserine (PS) and phosphatidylinositol (PIP2) in the absence of  $\text{Ca}^{2+}$ , attributed to the polybasic patch binding to PIP2/PS [6,9]. The presence of PIP2 leads to an increase in the  $\text{Ca}^{2+}$  affinity of Syt1, possibly due to conformational changes induced by PIP2 binding [6].  $\text{Ca}^{2+}$  binding by Syt1 also leads to a large decrease in the off-rate of Syt1 from membranes [9], presumably due to insertion of the nonpolar residues in the C2AB  $\text{Ca}^{2+}$  binding loops. A recent paper measured energetics of single-molecule C2AB interactions with optical tweezers in the presence of  $\text{Ca}^{2+}$  [20], but reported a lack of binding in the absence of  $\text{Ca}^{2+}$ . A previous single-molecule atomic force microscopy (AFM) study attached Syt1 to an AFM tip and performed adhesion force measurements at PC/PS membranes [21], but no binding energetics or distance-dependent Syt1-membrane interactions were reported. Additionally, while  $\text{Mg}^{2+}$  is known to bind in the Syt1  $\text{Ca}^{2+}$ -binding sites in the absence of  $\text{Ca}^{2+}$  [22] and has subtle effects on Syt1-membrane interactions [23,24], no studies have directly compared the binding energies in  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ . Therefore, there remains a lack of direct probes of the long-range and short-range behaviours and energetics of divalent ion-dependent Syt1 binding at anionic membranes.

We directly measured the interaction energy between a lipid membrane decorated with the cytoplasmic portion of Syt1 and an anionic membrane composed of PC/PS/PIP2 using a surface forces apparatus (SFA),



**Fig. 1.** A schematic of the SFA experiment. Syt1-coated membrane (top) interacts with an anionic membrane (bottom), consisting of 80 mol% POPC, 15 mol% DOPS and 5 mol% PIP2. The  $\text{Ca}^{2+}$  binding loops of C2A and C2B are indicated by the yellow sites, and the polybasic patch on C2B is indicated by the red site.

as shown schematically in Fig. 1. To isolate the Syt1-target membrane interaction, which has been shown to be more productive for fusion [5,24–26], we have included only PC and Syt1 in the protein-containing membrane. By using EGTA,  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  in the buffer, the effects of divalent ions are elucidated during Syt1 confinement, docking and unbinding at an anionic membrane surface, including binding energetics, kinetics and molecular rearrangements.

## Materials and methods

### Protein expression and purification

The DNA construct used in this study was generated by cloning the cytoplasmic domain (residues 83–421) of rat synaptotagmin-1 into pGEX6p-1 (GE Healthcare, Marlborough, MA, USA) using restriction sites XhoI and NotI. A 12x histidine residue tag was added upstream (N-terminus of the protein) using BamHI and XhoI. Two residues, C277A and E269C, were mutated using a QuikChange mutagenesis kit (Agilent Technologies, Santa Clara, CA,

USA) to allow for subsequent fluorescent labelling. The construct was transformed and grown in *Escherichia coli* BL21(DE3) to an OD 600 ~0.8 and the expression was induced with 0.5 mM IPTG. The cells were harvested after 4 h at 37 °C and suspended in lysis buffer (25 mM HEPES, pH 7.4, 500 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 15 mM Imidazole, 0.4 mM TCEP, 10% glycerol, 1% Triton X-100, protease inhibitors). The sample was lysed using a cell disrupter, and the lysate was supplemented with 0.1% polyethylimine before centrifugation (~96 000 *g* for 30 min). The supernatant was loaded onto nickel-nitrilotriacetic acid (Ni-NTA, Qiagen, Valencia, CA, USA) beads (3–4 h or overnight at 4 °C) with 10 µL of Benzonase (2000 units). The beads were washed with 20 mL of lysis buffer with 0.1% Triton X-100, then resuspended in 5 mL of lysis buffer supplemented with 10 µg·mL<sup>-1</sup> of DNase I, 10 µg·mL<sup>-1</sup> of RNaseA and 10 µL of Benzonase and incubated at room temperature for 1 h.

Subsequently, the beads were rinsed quickly with 10 mL of high salt buffer (25 mM HEPES, pH 7.4, 1M KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 15 mM Imidazole, 0.4 mM TCEP, 10% glycerol) to remove nucleotide contamination, and washed several times with 25 mM HEPES, pH 7.4, 500 mM KCl, 50 mM Imidazole, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.4 mM TCEP, 10% glycerol. The protein was eluted off the nickel beads in 25 mM HEPES, pH 7.4, 400 mM KCl, 500 mM Imidazole, 0.5 mM CaCl<sub>2</sub>, 0.4 mM TCEP, 10% glycerol. The GST tag was cleaved overnight at 4 °C using Precision protease, and then removed with a 1-h room temperature incubation in Glutathione-Sepharose (Thermo Fisher Scientific, Grant Island, NY, USA). The protein was then run on a size exclusion chromatography column (Superdex 75 16/60 High load) equilibrated with 25 mM HEPES, pH 7.4, 150 mM KCl, 0.4 mM TCEP and further purified by anionic exchange (Mono-S) chromatography. All chromatography was carried out with AKTA (GE Healthcare). The protein concentration was determined with a Bradford assay using BSA as a standard. The 260 nm/280 nm ratios were measured to check nucleotide contamination. The protein was flash frozen and stored at -80 °C with 20% glycerol.

### Surface forces measurements

The force–distance measurements were done with a home-built SFA similar to the original Israelachvili design [27]. Briefly, back-silvered mica surfaces were glued on cylindrical glass disks ( $R \sim 2$  cm) with UV-cured glue (NOA81, Norland Optics), then a monolayer of 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) was deposited on both surfaces at an area/molecule of 0.4 nm<sup>2</sup> using a home-built Langmuir–Blodgett trough [28]. DMPE binds strongly to mica, creating a stable inner monolayer on both surfaces. Next, on one surface we deposited an outer layer of 95% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

(POPC) and 5% 1,2-dioleoyl-sn-glycero-3-[(*N*-(5-aminocarboxypentyl)iminodiacetic acid)succinyl] nickel (DGS-NTA-Ni) with an area/molecule of 0.4 nm<sup>2</sup>, and on the other surface an outer layer of 80% POPC, 15% 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) and 5% L- $\alpha$ -phosphatidylinositol-4,5-bisphosphate (PIP2) was deposited at 0.5 nm<sup>2</sup>. The POPC/DOPS/PIP2 membrane was kept immersed in 25 mM HEPES, 150 mM KCl, with 0.5 mM of EGTA, and in certain cases, 0.5 mM of free Ca<sup>2+</sup> or 1.0 mM of free Mg<sup>2+</sup> buffer (calculated using Maxchelator, maxchelator.stanford.edu).

The 95% POPC, 5% DGS-NTA-Ni membrane was immersed in a small vial of the same buffer (~3 mL volume) into which ~5 µL of ~2 mg·mL<sup>-1</sup> 12xHis-Syt1 was injected and mixed well via pipet. After 1 h of protein immersion, the small vial was transferred twice into clean buffer solution (~200 mL volume) to remove unbound protein. Finally both surfaces were carefully transferred under buffer into the SFA chamber. One surface was mounted on a spring with the other on a stiff mount in a crossed-cylinder geometry. The distance was measured via multiple beam interferometry and the force by spring deflection. For each condition we measured at least three independent experimental setups, with at least eight independent contact locations and at least two different protein batches to demonstrate reproducibility. Error bars represent standard errors over the independent contact locations.

## Results

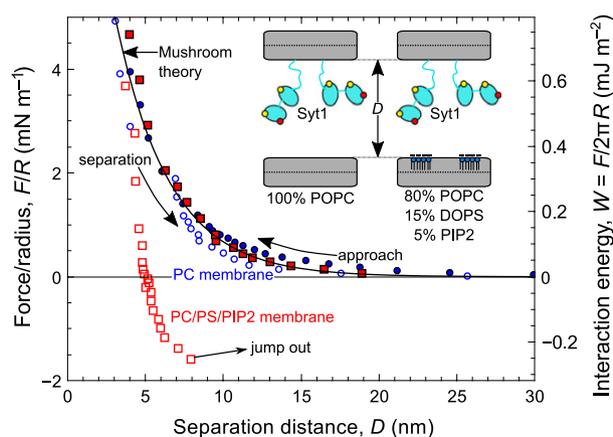
### Effect of anionic lipids on Syt1 interactions with lipid membranes

We measured the forces during approach and separation of a Syt1-decorated membrane and an anionic membrane. In the Syt1-membrane, accounting for 5% Ni-NTA and 2–3 histidines binding per Ni-NTA gives a maximum surface density of  $\sim 2.5 \times 10^{16}$  molecules of Syt1 per m<sup>2</sup> corresponding to  $\sim 40$  nm<sup>2</sup>/Syt1 molecule or  $\sim 1.3$  Syt1 copies per every 100 lipids in the outer leaflet. As such, the experiment simulates a synaptic vesicle approaching the anionic plasma membrane, albeit in the absence of SNAREs and other regulatory proteins, in order to isolate the pure Syt1-membrane interactions.

An SFA measurement consists of approaching and separating the surfaces while measuring the distance interferometrically (distance resolution  $\sim 1$  Å) and measuring the corresponding forces with a cantilever spring (force resolution  $\sim 100$  nN). In the standard procedure, we apply a 1 h contact time ( $t_c = 1$  h) between the end of the approach and the beginning of the separation, with shorter  $t_c$  in specific cases. The surfaces are approached and separated quasi-statically, such that the

distance and force are measured simultaneously every  $\sim 10$  s. The average speed of approach/separation outside of the interaction zone (i.e.  $D > 50$  nm) is  $\sim 1$  nm·s $^{-1}$ , which captures the quasi-equilibrium interaction force profile between Syt1 and the opposing membrane. The surfaces were initially immersed in 150 mM KCl, 25 mM HEPES buffer and 0.5 mM EGTA. To first examine the effects of anionic lipids on the binding of Syt1 to membranes, we adjusted the composition of the opposing ‘target’ membrane. We measured the force–distance interactions of Syt1 approaching an anionic (PS/PIP2) membrane surface, and compared these interactions with Syt1 approaching a neutral (100% POPC) membrane surface (Fig. 2). By convention, repulsive forces have a positive sign while attractive forces have a negative sign.

During the approach of Syt1, a significant force was first measured at  $D \sim 25$ – $30$  nm, which roughly corresponds to the fully stretched Syt1 [29]. Electrostatic interactions at this range are vanishingly small (Debye length  $\sim 0.8$  nm), so this interaction is attributed to a steric interaction between the Syt1 chains and the anionic membrane surface. As the surfaces are pushed further together (a primitive mimic of the SNARE/Munc13 complex which brings the vesicle and plasma membrane together from large distances), an exponential repulsion is measured. These observations indicate that in spite of the structured C2A and C2B domains, the 61-residue linker between the transmembrane domain and C2A combined with the 9-residue linker between C2A and C2B



**Fig. 2.** Interaction force vs. distance measurement (approach and separation) by SFA for Syt1 membrane with a 100% POPC membrane (blue circles), and a Syt1-coated membrane with an 80% POPC, 15% DOPS and 5% PIP2 membrane (red squares). Filled symbols are during approach while empty symbols are during separation. In the Syt1-membrane cartoons (upper right), the Ca $^{2+}$  binding loops of C2A and C2B are indicated by the yellow sites, and the polybasic patch on C2B is indicated by the red site.

are unstructured when not under confinement, and the isolated protein behaves similar to a random coil. We therefore apply the polymer mushroom model, which has also been applied to SNARE proteins [30],

$$F/R = 72\pi\Gamma k_B T e^{-\sqrt{3}D/R_g}, \quad (1)$$

where  $F$  is the measured force,  $R \sim 2$  cm is the radius of the surfaces,  $\Gamma$  is the surface density of Syt1,  $k_B$  is Boltzmann's constant,  $T = 298$  K is the temperature,  $D$  is the distance between the membranes (see Fig. 1) and  $R_g$  is the radius of gyration of Syt1. By fitting this equation to the measured  $F/R$  vs.  $D$  curves, we measure  $\Gamma$  and  $R_g$ .

The approach curves (filled points, Fig. 2) are nearly equivalent for both the PC membranes and the anionic membranes, revealing that the long-range interactions between Syt1 and membranes do not depend on the membrane charge. These repulsive forces follow the mushroom model closely, and the measurement reveals a surface coverage  $\Gamma \sim 1 \times 10^{16}$  molecules·m $^{-2}$  and radius of gyration  $R_g \sim 6 \pm 0.5$  nm for both conditions (Fig. 2, black curve). However, upon separating the two surfaces, drastic differences are observed between the PC lipids and anionic lipid membranes. For the PC membrane, a small hysteresis is measured (compared to the approach curve), but no adhesion is observed. This suggests that the Syt1 molecules are in a slightly compressed mode upon separation, but they do not bind specifically to the PC lipids. Conversely, for the anionic membrane, a strong adhesion force  $F_{ad}$  is measured, as the force at which the spring experiences an instability and a so-called ‘jump-out of contact’ is observed (indicated by the ‘jump-out’ arrow in Fig. 2). By applying Derjaguin's approximation, which is valid for  $R \gg D$  and if the interactions decrease at least as  $1/D^2$  (and when the surfaces are not deformed or flattened), we find the surface energy per unit area,  $W$ , as

$$W = \frac{F}{2\pi R} \quad (2)$$

The normalized adhesion force is measured as  $F_{ad}/R = -1.6$  mN/m, corresponding to an adhesion energy  $W_{ad} = -0.25$  mJ·m $^{-2}$ . Then, using this adhesion energy, one can find the energy per molecule of Syt1, by

$$E_{Syt1} = W/\Gamma \quad (3)$$

As such, we measure the energy per molecule as a function of distance between the membranes, allowing for distance-dependent probing of the energetics of confinement and binding of Syt1. In the case of Syt1 binding to the anionic lipid membrane in 0.5 mM EGTA,  $W_{ad} = -0.25$  mJ·m $^{-2}$  and  $\Gamma = 1.3 \times 10^{16}$  molecules·m $^{-2}$  give a binding energy  $E_{Syt1} \sim 5 k_B T$ .

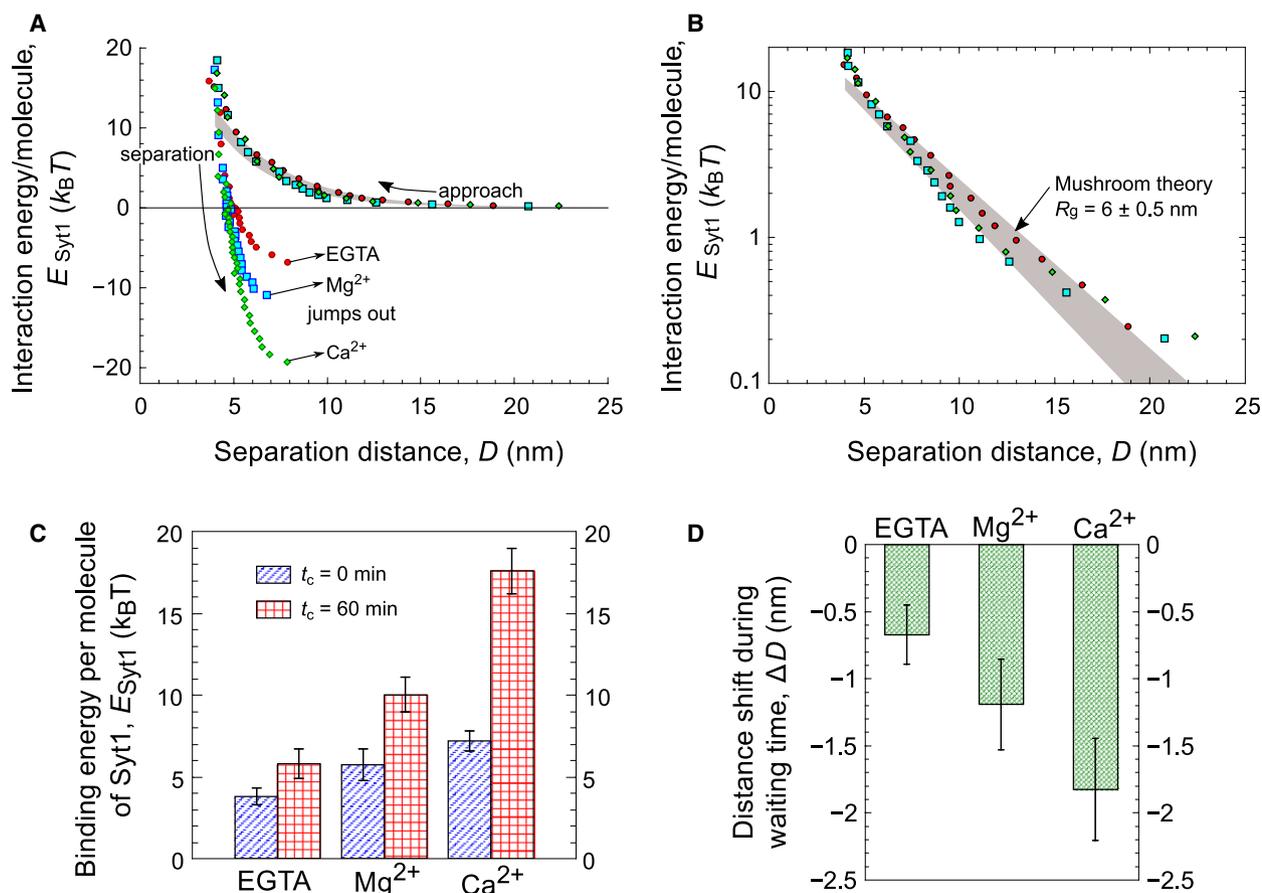
These values were taken from the force–distance measurement presented in Fig. 2. Calculating the average and standard error over a representative sample of eight independent measurements gives  $E_{\text{Syt1}} = 5.8 \pm 0.9 k_B T$  in EGTA. Note that the sign of the binding energy is reported as positive by convention as the magnitude of the adhesion (i.e. negative) force and energy.

### Effects of divalent cations $\text{Mg}^{2+}$ and $\text{Ca}^{2+}$ on Syt1 interactions with lipid membranes

Next, we measured energy vs. distance curves separately in 0.5 mM EGTA, 0.5 mM free  $\text{Mg}^{2+}$  and 0.5 mM free  $\text{Ca}^{2+}$ , to examine the effects of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  on the interaction between Syt1 and the anionic membrane (Fig. 3). As Syt1 approaches the membrane, an exponential repulsive force profile was observed again (Fig. 3A,B), which closely follows the mushroom model with  $R_g = 6 \pm 0.5$  nm in all three cases (Fig. 3B), indicating that the divalent ions do

not have a significant effect on the extended structure before Syt1 contacts the membrane. The surfaces are driven together until  $F/R \sim 5$  mN/m, where the distance remaining between the bilayers is  $D = 5.2 \pm 0.3$  nm (i.e. the confined Syt1 thickness), a similar value to thicknesses previously measured for Syt1 bridging between liposomes [8,10,29,31]. As observed by the repulsive interactions (Fig. 3A,B), confining Syt1 to this level has an energetic cost of  $\sim 15\text{--}20 k_B T$ . This energy might be overcome by binding to SNAREs or Munc13 *in vivo*, or if Syt1 localizes away from the centre of the contact zone of the highly curved vesicle, then this barrier could be significantly lower.

Figure 3A,B shows selected force runs; every individual measurement results in a measurement of  $\Gamma$ ,  $R_g$ ,  $W_{\text{ad}}$  and therefore  $E_{\text{Syt1}}$ . Typically,  $\Gamma$  ranges from about  $\sim 5 \times 10^{15}$  to  $\sim 2 \times 10^{16}$  molecules·m $^{-2}$ , while  $R_g$  is in the range 5–8 nm. When the surfaces are separated from each other, divalent ions lead to significant increases to the binding energy (Fig. 3A,C). Addition



**Fig. 3.** Force–distance measurements by SFA in the presence of EGTA,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  for (A) approach and separation on normal axes and (B) approach forces only on a semilog plot. (C) Summary of binding interactions at short and long contact times and (D) summary of the distance shift during the 1-h contact time for each condition.

of  $\text{Ca}^{2+}$  is known to cause insertion of the hydrophobic residues near the  $\text{Ca}^{2+}$  binding sites of C2AB, and the binding energy increases to  $E_{\text{Syt1}} = 17.6 \pm 1.4 k_{\text{B}}T$ . Notably, the binding increases in  $\text{Mg}^{2+}$ , from  $E_{\text{Syt1}} = 5.8 \pm 0.9 k_{\text{B}}T$  in EGTA to  $E_{\text{Syt1}} = 10.0 \pm 1.1 k_{\text{B}}T$  in  $\text{Mg}^{2+}$ . This increase in the binding energy implicates interaction of the hydrophobic loops when Syt1 coordinates  $\text{Mg}^{2+}$ , possibly due to a weaker hydrophobic loop interaction compared to  $\text{Ca}^{2+}$  (see discussion for details). A subset of experiments were performed in buffer containing 0.5 mM free  $\text{Ca}^{2+}$  and 1 mM free  $\text{Mg}^{2+}$  over two independent experimental setups, eight total contact zones and three individual force measurements at each contact zone. These measurements gave nearly identical results to the energy measured in  $\text{Ca}^{2+}$  alone, with  $E_{\text{Syt1}} = 16.1 \pm 2.1 k_{\text{B}}T$ .

The binding energies at short contact times,  $t_{\text{c}} = 0$  min, were also measured, as shown in Fig. 3C. These results show the same trend as the binding energies for  $t_{\text{c}} = 60$  min, although the trend is less pronounced. The experimental subset performed in 0.5 mM free  $\text{Ca}^{2+}$  and 1 mM free  $\text{Mg}^{2+}$  gave  $E_{\text{Syt1}} = 7.9 \pm 0.9 k_{\text{B}}T$  for  $t_{\text{c}} = 0$  min, again virtually the same as the value measured in  $\text{Ca}^{2+}$  alone ( $E_{\text{Syt1}} = 7.2 \pm 0.6 k_{\text{B}}T$ ). The binding kinetics, which plateau between  $t_{\text{c}} = 30$ –60 min, are shown in supporting information (Data S1). Syt1 triggers  $\text{Ca}^{2+}$ -dependent fusion remarkably quickly *in vivo*, often in less than a millisecond [2]. The slow equilibration in the SFA measurement, over the course of  $\sim 1$  h, is clearly nonbiological. This timescale for equilibration was also observed for SNARE proteins [30] and is likely due to the surface geometry, which provides confinement to nm-level distances over hundreds of  $\mu\text{m}^2$  compared to hundreds of  $\text{nm}^2$  in the synaptic vesicle. However, the slow kinetics are in fact advantageous in the present measurements because it enables observation of slow molecular rearrangements. This confinement effect may be enhanced by the Syt1 concentration which is around 10x larger than the average Syt1 content of a synaptic vesicle [32]. Nonetheless, if all Syt1 are bound to the plasma membrane, e.g. as in the recently proposed ring-shaped oligomers model [19], their local concentration is increased up to the same order of magnitude as the concentration in the current SFA experiments. In any case, using 10x smaller Syt1 concentration in the present measurements would decrease the magnitudes of  $\Gamma$  and  $W_{\text{ad}}$  accordingly, making accurate measurement of  $E_{\text{Syt1}}$  impossible.

By measuring the distance at the final point of approach and first point of separation at the same applied force, we obtain the distance shift during the contact time,  $\Delta D = D_{\text{separation,initial}} - D_{\text{approach,final}}$ , as shown in detail in the supporting information (Data

S2). A negative value for  $\Delta D$  indicates an inward distance shift (i.e. the surfaces become closer together). For  $t_{\text{c}} = 0$  min,  $\Delta D$  is zero within experimental error. However, for  $t_{\text{c}} = 1$  h,  $\Delta D$  increases in magnitude from  $-0.7$  nm in EGTA, to  $-1.2$  nm in  $\text{Mg}^{2+}$ , and to  $-1.8$  nm in  $\text{Ca}^{2+}$ , as shown in Fig. 3D. Therefore, the binding energies were correlated with  $\Delta D$ , indicating that relatively slow molecular rearrangements during confinement lead to the increased adhesion, discussed in more detail below.

## Discussion

Most previous measurements of Syt1-membrane binding have utilized only the soluble C2AB to measure association with a membrane. Importantly, we include the entire cytoplasmic portion of Syt1 and attach it directly to a membrane, providing a more precise mimic of Syt1 in synaptic vesicles approaching the plasma membrane. Under physiological conditions, the Syt1 interaction with *cis* PS lipids is known to be screened by ATP, such that only the *trans* interaction is productive [26]. Therefore, by including only PC in the *cis* membrane, we measure the Syt1-*trans* interaction under the physiologically relevant topology.

Previous solution phase measurements indicate that  $\text{Ca}^{2+}$ -independent binding of C2AB to anionic membranes occurs through the C2B polylysine patch, while the C2A plays a small or negligible role in lipid binding [5,7,23]. Therefore, the adhesion measured here between Syt1 and the anionic membrane in EGTA,  $E_{\text{Syt1}} = 5.8 \pm 0.9 k_{\text{B}}T$ , likely arises primarily from the binding of the polylysine patch of C2B with the anionic PS and PIP2 lipid headgroups. Several groups have measured association or dissociation constants via isothermal calorimetry or MST, which can be used to calculate a binding free energy. With similar lipid compositions the dissociation constant measurements of Syt1 with membranes are reported to be in the range of  $\sim 5$ – $10 k_{\text{B}}T$  in EGTA [6,7]. A recent single-molecule study reported no binding between C2AB and an anionic membrane in the absence of  $\text{Ca}^{2+}$ , possibly because the  $\text{Ca}^{2+}$ -independent binding requires participation of multiple molecules [20]. The value reported here ( $5.8 \pm 0.9 k_{\text{B}}T$ ) is in the lower range of values reported thus far, which is perhaps expected. By including the entire cytosolic domain and anchoring it to a membrane, we have reduced degrees of freedom compared to the solution phase measurements. Similarly, the extreme confinement due to the close apposition of both membranes reduces the degrees of freedom for Syt1 even further and provides a model closer to the crowded and confined situation *in vivo*.

A simple screened Coulomb interaction to model the polybasic patch as a cation of valence +4 and the PIP2 as an anion of valence  $-3$  allows for a simplified view of this interaction. As shown in the supporting information (Data S3), this ion–ion interaction is fully attractive but becomes significant compared to the thermal energy only for separations  $\sim 1$  nm. The contact energy (i.e. adhesion or binding energy) is  $\sim 8 k_B T$ . A combined experimental–theoretical study of polylysine binding at anionic membrane surfaces found that each lysine provided about  $1.7 k_B T$  to the total binding energy [33], such that a 4-lysine stretch as found in C2B should bind with  $\sim 7 k_B T$ . These simplified models are in rough agreement with the experimentally measured value, indicating that the C2B–membrane interaction is almost purely accounted for as a charge–charge interaction between the polybasic patch and the anionic lipids.

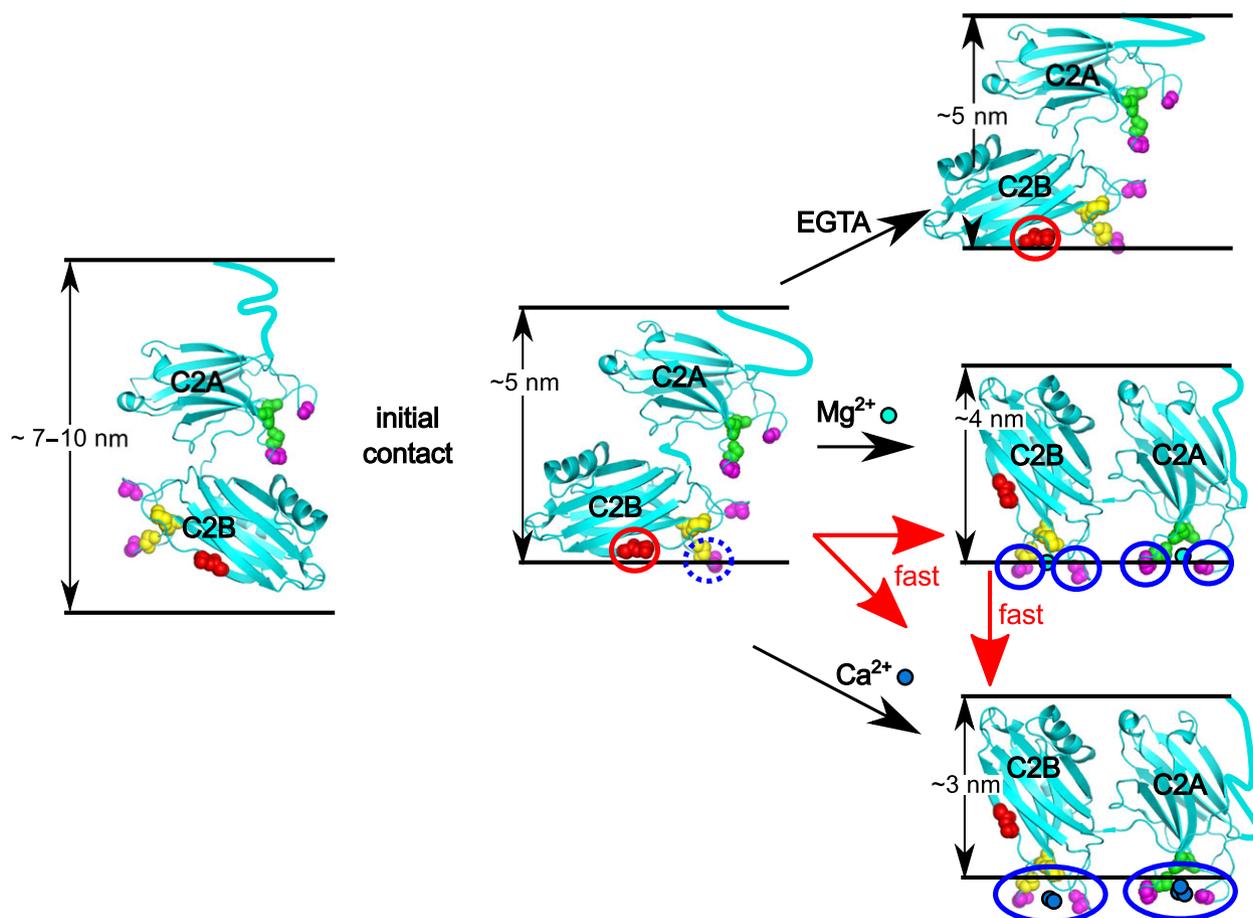
The increased binding of Syt1 in the presence of  $\text{Ca}^{2+}$  is also expected from previous work [9,20], which indicates that Syt1 reorients to insert the hydrophobic residues of the  $\text{Ca}^{2+}$  loop into the membrane. A recent optical tweezers study reported a binding energy of  $\sim 12 k_B T$  in the presence of  $0.5$  mM free  $\text{Ca}^{2+}$  for C2AB binding to an anionic membrane [20]. A wide range of dissociation constants, and therefore binding energetics, have been measured by traditional biological assays for soluble C2AB with anionic membranes, with a maximum value of  $\sim 25 k_B T$  [9]. The value measured here in  $0.5$  mM  $\text{Ca}^{2+}$ ,  $E_{\text{Syt1}} = 17.6 \pm 1.4$ , is again smaller than the traditional assays, although is potentially more representative physiologically because Syt1 is anchored to the membrane.

The increase in the binding energy upon addition of  $\text{Mg}^{2+}$  (compared to in EGTA) has not been previously reported and is potentially due to a weaker interaction of the  $\text{Ca}^{2+}$  binding pocket in the presence of  $\text{Mg}^{2+}$  that results in partial insertion of the hydrophobic loops. The membrane binding energy in  $\text{Mg}^{2+}$  is between the binding energies measured in  $\text{Ca}^{2+}$  and EGTA. One possible explanation for the weaker overall interaction in  $\text{Mg}^{2+}$  compared to  $\text{Ca}^{2+}$  is that partial hydrophobic loop insertion occurs in concert with binding of the polybasic patch. The other possibility is that the polybasic patch is no longer bound in the presence of  $\text{Mg}^{2+}$  and the binding energy originates entirely from the partial loop insertion. These possibilities are presented schematically in Fig. 4 and discussed further directly below. We cannot distinguish between these in the current measurements and more detailed structural measurements could help elucidate the mechanism. Since we obtained similar binding energies for experiments in  $\text{Ca}^{2+}$  and both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$

simultaneously, it appears that the  $\text{Mg}^{2+}$  occupies the  $\text{Ca}^{2+}$  sites in the absence of  $\text{Ca}^{2+}$ , but  $\text{Ca}^{2+}$  outcompetes  $\text{Mg}^{2+}$  when they are both present, such that Syt1 reaches the full  $\text{Ca}^{2+}$ -dependent binding energy.

Due to the geometry in the SFA, the first Syt1–membrane interaction is through the C2B polybasic patch which orients the C2B and provides a similar initial binding conformation in all three conditions, as shown in Fig. 4. The measurements of  $\Delta D$  indicate that Syt1 molecules slowly rearrange to find their final conformation. Even for  $t_c = 0$  min, the surfaces remain at  $\sim 5$  nm level confinement for  $\sim 5$  min, allowing some small fraction of molecules to change conformation and leading to the observed adhesion increase for the  $t_c = 0$  min case over the three conditions. In EGTA, the small increase in binding energy over  $t_c = 1$  h combined with the small value for  $\Delta D$  implicate nonspecific rearrangements. In  $\text{Ca}^{2+}$ , the C2B hydrophobic loops prefer an upright orientation during loop insertion, allowing C2A to rotate and align parallel with the C2B during the equilibration to insert its hydrophobic loops in the membrane, leading to the measured large increase in binding and more dramatic rearrangements of  $\Delta D = -1.8$  nm in  $\text{Ca}^{2+}$ . With  $\text{Mg}^{2+}$ , the C2B again contacts the membrane first via the C2B polybasic patch, with the additional possibility of partial loop insertion. Rotation during the contact time results in a similar side-by-side configuration of C2AB, but due to only partial hydrophobic loop insertion, the distance shift is limited to  $\Delta D = -1.2$  nm and the binding energy increase is modest.

With a physiological concentration of  $\sim 1$  mM  $\text{Mg}^{2+}$ , the measurements in  $\text{Mg}^{2+}$  potentially reveal mechanisms of Syt1 action. Several previous studies have suggested that the presence of  $\text{Mg}^{2+}$  is important for the  $\text{Ca}^{2+}$  sensitivity of Syt1 [22–24]. Rearrangements of potential physiological importance measured here include the transition from polybasic patch binding, to partial loop insertion in  $\text{Mg}^{2+}$ , to full loop insertion in  $\text{Ca}^{2+}$  (Fig. 4). Additionally, if C2A is held adjacent to the *cis* membrane by SNAREs or other conformational factors, the rearrangement from C2B polybasic binding/partial loop insertion in  $\text{Mg}^{2+}$  to full loop insertion in  $\text{Ca}^{2+}$  may be physiologically relevant. These rearrangements are not necessarily sequential and might be multimodal such that during loop insertion, the polybasic patch interaction persists to some degree [9]. The physiological roles of these  $\text{Mg}^{2+}$  effects merit further exploration in a functional context. While these measurements are consistent with the ring model [16,18,19], no direct evidence of Syt1 rings or oligomers was found. It is difficult to envision how oligomerization would impact the measured results in



**Fig. 4.** Schematic mechanism for the Syt1-membrane rearrangements measured in this study. The Syt1 is anchored to the top membrane. Important residues of Syt1 are labelled as follows: red, C2B polybasic; yellow, C2B  $\text{Ca}^{2+}$  binding; green, C2A  $\text{Ca}^{2+}$  binding; pink, C2A and C2B hydrophobic loops. Membrane contacts are denoted by red circles (polybasic patch), blue circles (partial hydrophobic loop insertion), blue ovals (full hydrophobic loop insertion). Black arrows denote the slow measured rearrangements (over the course of 1 h), while hypothesized fast (~msec) potential physiological transitions are denoted by the heavy red arrows (labelled 'fast'). See text for detailed mechanistic explanation.

SFA. While the slow rearrangements might reflect oligomerization, they also simply might be an effect of the large scale confinement over many  $\mu\text{m}^2$ . Similarly, the measured  $R_g$  is  $\sim 2\text{--}3\times$  larger than the expected  $R_g$  for Syt1 [34,35], which could be a subtle signature of oligomerization, but also may result from differences between measuring  $R_g$  for the full cytoplasmic domain between two surfaces (as done here) vs. measuring  $R_g$  of C2AB in solution. The transition from the initial contact state in  $\text{Mg}^{2+}$  to the equilibrated loop insertion in  $\text{Ca}^{2+}$  is consistent with the ring model.

We directly measured Syt1-membrane binding energies and interaction mechanisms, along with confinement and molecular rearrangement details of Syt1-membrane interactions. Future measurements focusing on Syt1 mutants and more realistic lipid

compositions will help to precisely assess the roles of different Syt1 binding sites. While the current results suggest that the hydrophobic loops play a role in the presence of  $\text{Mg}^{2+}$ , additional structural and biochemical work is required to elucidate the precise nature of this interaction. The inclusion of SNAREs and observation of Syt1 loop-insertion, in correlation with measurements of distance-dependent binding energetics, could help elucidate precise mechanistic details of Syt1 action in fast  $\text{Ca}^{2+}$ -triggered synaptic transmission.

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## Author contributions

SHD, FP, and JER conceived and designed the study. CG and SHD performed experiments. CG, JC, OB, SSK, and EP provided new tools and reagents. CG, SHD, and FP analyzed data and wrote the paper. All authors discussed the results and revised the manuscript.

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## Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Data S1.** Increase in binding energy during contact time.

**Data S2.** Measurement of distance shift during waiting time.

**Data S3.** Screened Coulomb interaction between polylysine patch and PIP2.