# Doc2b is a High Affinity Ca2+ Sensor for Spontaneous Neurotransmitter Release

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## Abstract

Synaptic vesicle fusion in brain synapses occurs in phases that are either tightly coupled to action potentials (synchronous), immediately following action potentials (asynchronous) or as stochastic events in the absence of action potentials (spontaneous). Synaptotagmin-1, -2 and -9 are vesicle-associated Ca$^{2+}$-sensors for synchronous release. Here we found that Double C2 domain (Doc2) proteins act as Ca$^{2+}$-sensors to trigger spontaneous release. Although Doc2 proteins are cytosolic, they function analogously to synaptotagmin-1 but with a higher Ca$^{2+}$-sensitivity and superior in vitro fusion-efficiency. Doc2 proteins bound to SNARE-complexes in competition with synaptotagmin-1. Thus, different classes of multiple C2 domain-containing molecules trigger synchronous versus spontaneous fusion, which suggests a general mechanism for synaptic vesicle fusion triggered by the combined actions of SNAREs and multiple C2 domain-containing proteins.

Neurotransmitter release is triggered by a rise in intracellular Ca$^{2+}$, which activates sensors that subsequently trigger vesicle fusion. Synchronous release, the fastest mode of neurotransmission, involves the Ca$^{2+}$ sensors synaptotagmin-1, -2 or -9 which are anchored in the vesicle membrane and contain two cytoplasmic C2 domains that bind phospholipids in a Ca$^{2+}$-dependent manner and interact with the soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) complex (1-5). Synaptotagmin-1-deficient neurons lack synchronous release but display an increase in spontaneous release (6-9) except in autapses (1, 10), suggesting a distinct mechanism for spontaneous release. Spontaneous release occurs in the absence of action potentials and is largely Ca$^{2+}$-dependent (12-16), although truly Ca$^{2+}$-independent fusion may also exist (11).

Doc2a and Doc2b are soluble proteins that contain C2 domains with high similarity to synaptotagmins (17). They are expressed in nerve terminals and interact with the secretory

molecules Munc18, Munc13 and the SNARE proteins syntaxin-1 and SNAP25 (18, 19). Overexpression of Doc2b enhances exocytosis in chromaffin cells (18), pancreatic beta cells (20, 21) and adipocytes (22) but its function in neurons is elusive.

## Role of Doc2b and Ca²⁺ in spontaneous synaptic release

We generated $Doc2b^{-/-}$ mice by deleting the promoter and exon 1 of the $Doc2b$ gene (fig. S1) (23). $Doc2b^{-/-}$ mice did not express the remaining exons and lacked Doc2b immunoreactivity. $Doc2b^{-/-}$ mice were viable and fertile without gross abnormalities. Other proteins implicated in neurotransmitter secretion were expressed at normal levels (fig. S1D). Compensatory ectopic expression of $Doc2a$ was not detected in Doc2b-deficient brains by in situ hybridization and the Doc2a protein level was unchanged (fig. S1). $Doc2a^{-/-} Doc2b^{-/-}$ double knock-out (DKO) mice were also viable, fertile and indistinguishable with regard to gross anatomy. To study neurotransmission and synaptic plasticity in Doc2 mutants, hippocampal neurons were cultured on glial microislands to promote self-innervation (autapses). Because Doc2a and Doc2b are both expressed in the hippocampus (17), DKO mice were compared to wild-type controls. All aspects of evoked release were normal, with excitatory postsynaptic currents (EPSC) of normal amplitude and shape (Fig. 1A), a normal synaptic depression during prolonged stimulation at 5 or 40 Hz (Fig. 1B and fig. S2A,B), a normal contribution to the total postsynaptic charge transfer (24) of asynchronous release (Fig. 1B) and a normal size of the readily releasable pool (RRP, fig. S2D).

However, DKO mice exhibited a twofold reduction in the spontaneous release frequency (6.2 Hz in wild-type to 3.3 Hz in DKO; Fig. 1C,D). Acute expression of Doc2b in DKO neurons restored this phenotype. Doc2b immunoreactivity was confirmed in all cellular compartments including synapses, before the start of recordings (fig. S3). As a control, acute expression of green fluorescent protein (GFP) in DKO cells did not affect the spontaneous release frequency (3.1 Hz, Table S1). A normal organization of the postsynaptic apparatus was indicated by a normal mEPSC amplitude, rise time and decay time in DKO neurons (fig. S2F) or after overexpression (fig. S3B).

Because Doc2 proteins require $Ca^{2+}$ for their phospholipid-binding and secretion enhancing activity (18, 25), we investigated if spontaneous release also depended on $Ca^{2+}$. The spontaneous release frequency was measured in the same cells before and after loading them with the $Ca^{2+}$-chelator BAPTA. This significantly reduced the frequency to 2.0 Hz (p<0.0001) in wild-type cells and to 1.4 Hz in DKO cells (p<0.0001, Fig. 1E,F). The frequency did not decrease further upon longer incubation with BAPTA-AM (fig. S2H). Thus, as expected (13-16), spontaneous events are often triggered by short-lived $Ca^{2+}$ transients in resting cells.

Consistent with the role of $Ca^{2+}$ in spontaneous release, the frequency of individual release events markedly increased after intense activity (5 Hz for 20 s; Fig. 1G,H) and decayed after the last stimulus with an exponential halftime of 11 s. Similar observations have been made in the neuromuscular junction and calyx of Held (26, 27). In DKO cells after intense stimulation the number of observed events was reduced to 50% of wildtype, except for the first time point that was sampled 200-1200 ms after the last depolarizing stimulus, where asynchronous release may still contribute (Fig. 1H). The Doc2-independent events remaining in DKO cells were also affected by BAPTA and repetitive firing, suggesting that additional $Ca^{2+}$-dependent mechanisms exist besides Doc2a/b-dependent events. Thus Doc2 proteins responding to intracellular $Ca^{2+}$ are required for approximately half of the spontaneous release events in hippocampal neurons.

## Disinhibition in Purkinje cells lacking Doc2b

*Doc2b* mRNA was abundant in Purkinje cells (PCs) of the cerebellum (Fig. 2A) while *Doc2a* was not detectable. *Doc2b* expression was exclusive to the PC layer with no detectable mRNA in other cerebellar cells including interneurons. PCs synapse onto neighboring PCs via recurrent axon collaterals (28). We performed whole cell voltage-clamp recordings at postnatal days 7-8 because at this time, recurrent synapses are the predominant source of GABAergic input while stellate and basket cells are still functionally immature (29). In the presence of 6,7-dinitroquinoxaline-2,3-dione (DNQX) to block AMPA receptors and tetrodotoxin (TTX) to block sodium currents, the PCs had a stable resting potential without NMDA receptor currents or $Ca^{2+}$-spikes. Under these conditions, any remaining inhibitory postsynaptic currents (IPSCs) can be interpreted as spontaneous release events.

The frequency of spontaneous IPSCs was reduced fourfold in $Doc2b^{-/-}$ mice compared to control littermates (Fig. 2B,C). Postsynaptic parameters were normal (i.e. amplitude, rise time and decay). In young rodents, GABAergic input inhibits PC firing (29) and recurrent PC-PC synapses are the major GABA source (30). We thus tested if the disinhibition in $Doc2b^{-/-}$ PC-PC synapses affected PC spiking. Whole cell current clamp recordings were performed at postnatal day P17 in the absence of DNQX or TTX. We observed various firing patterns in PCs from wild-type or heterozygous mice: irregular trains of simple spikes in most cells (Fig. 2D) or trimodal firing patterns comprised of tonic, burst and silent periods, as expected (30, 31). In contrast, PCs from $Doc2b^{-/-}$ mice showed continuous spiking without interruptions (Fig. 2E). This pattern occurred with a frequency of 20-25 Hz and was observed in all $Doc2b^{-/-}$ cells tested, but never in control cells.

We also investigated neurotransmission in the calyx of Held synapse, a giant glutamatergic synapse in the auditory brainstem where the Doc2-Munc13 interaction is suggested to contribute to presynaptic plasticity (32). DKO mice exhibited normal evoked responses with a 2-fold lower spontaneous release frequency (1.9 Hz compared to 4.8 Hz in control littermates), but this difference did not reach statistical significance (fig. S4).

## Phospholipid- and SNARE interactions

Doc2b is structurally similar to synaptotagmin-1 but lacks a transmembrane domain and its C2 domains have a relatively high $Ca^{2+}$ affinity (25). It is thus conceivable that Doc2b mediates spontaneous release events similar to synaptotagmin-dependent synchronous release, only with slower kinetics and a higher $Ca^{2+}$-sensitivity. We thus tested if Doc2b is biochemically similar to synaptotagmin-1.

Doc2b C2A domain bound in a $Ca^{2+}$-dependent manner to phosphatidylserine-containing membranes (Fig. 3A) (33, 34). In addition, the C2B domain showed weak $Ca^{2+}$-dependent phospholipid binding (Fig. 3A). The C2AB fragment of Doc2b showed stronger liposome binding than the isolated C2A and C2B domains (Fig. 3A). $Ca^{2+}$-dependent membrane binding by synaptotagmin-1 was enhanced in the presence of phosphatidyinositol(4,5)bisphosphate ($PIP_2$) (35). For the C2A and C2B domains of Doc2b the inclusion of $PIP_2$ enhanced both $Ca^{2+}$-dependent and -independent binding (Fig. 3B). This $Ca^{2+}$-independent binding was localized to two polybasic patches on the C2A and C2B domains, respectively (figs. S5 and S6). The use of brain-derived Folch lipids (36) generally increased binding, including the $Ca^{2+}$-independent binding by the C2A domain (Fig. 3C, figs. S5 and S6). The C2AB domains of Doc2a and synaptotagmin-1 displayed similar behavior (Fig. 3D).

The C2AB domain of synaptotagmin-1, -3 and -5 induce a high degree of positive membrane curvature in a $Ca^{2+}$-dependent manner (3), due to the insertion of the tips of both

C2 domains into the hydrophobic phase of the membrane. Shallow insertions into a monolayer are known to induce bending of the membrane towards the insertion (37-39). In order to examine if Doc2b also induces membrane curvature, we incubated liposomes derived from Folch lipids with 10 μM Doc2b C2AB domain in the absence (EDTA) or presence of $Ca^{2+}$ (Fig. 3E). Indeed, the Doc2b C2AB domain efficiently induced tubulation of otherwise spherical liposomes in a strictly $Ca^{2+}$-dependent manner (Fig. 3E). We frequently observed closely aligned tubules indicating that the Doc2b C2AB domain could cluster liposomes in addition to their tubulation. Tubulation was still apparent at a Doc2b concentration of 5 μM but was barely detectable below 1 μM.

Synaptotagmin-1 requires SNARE complex binding to promote fusion in vitro and in vivo (4, 5). We thus tested if Doc2b also bound SNARE complexes. In one set of experiments we assembled the SNARE core complex on glutathione beads using the synaptobrevin SNARE domain fused to GST (fig. S7). The Doc2b and synaptotagmin-1 C2AB domains were subsequently added in the presence or absence of $Ca^{2+}$. In another set of experiments we used the GST-Doc2b C2AB domain to pull down the soluble SNARE complex (Fig. 4A). As expected (40), we found efficient $Ca^{2+}$-dependent binding of the synaptotagmin-1 C2AB domain to SNARE complexes (fig. S7). The Doc2b C2AB domain also bound to SNARE complexes (Fig. 4A and fig. S7). In the C2B domain of synaptotagmin-1 the same poly-lysine motif that binds $PIP_2$ mediates SNARE complex binding (35). We thus tested SNARE complex binding by the mutant $C2AB^{K237,319E}$ domain of Doc2b. As expected, it showed a profound loss of SNARE complex binding (Fig. 4A and fig. S7). Next we tested if the putative membrane inserting hydrophobic amino acids in the tips of the $Ca^{2+}$-binding loops (Fig. 4A, fig. S7) contributed to SNARE complex binding. To this end we mutated H158, F222 and I360A. The first $Ca^{2+}$-binding loop of the Doc2b C2B domain contains an alanine and so was not mutated. We refer to this mutant as the 4A mutant. It showed no defect in SNARE complex binding indicating that, as for synaptotagmin-1, SNARE complex and membrane binding can occur at the same time (Fig. 4A and fig. S7). In contrast, $Ca^{2+}$-dependent liposome binding was only affected by the 4A but not the K237, 319E mutation (Fig. 4C). The decreased binding of the 4A mutant was less effective if the liposomes were made with higher percentages of phosphatidylserine.

The high structural and biochemical similarities between Doc2b and synaptotagmin-1 suggest that the binding site on the SNARE complex may be identical. We thus tested if the C2AB domain of synaptotagmin-1 competes with the C2AB domain of Doc2b for SNARE complex binding (Fig. 4B). Indeed, increasing amounts of the synaptotagmin-1 C2AB domain competed off the Doc2b C2AB domain suggesting that the binding sites for synaptotagmin-1 and DOC2B are overlapping.

## $Ca^{2+}$-dependent promotion of membrane fusion by Doc2b

The C2AB domains of several members of the synaptotagmin family promote fusion of liposomes containing reconstituted SNAREs in a $Ca^{2+}$-dependent manner (41). Similarly, the C2AB domain of Doc2b promoted fusion of SNARE-containing liposomes and this activity was strictly $Ca^{2+}$-dependent (Fig. 4D-H). When $Ca^{2+}$ was introduced at a later time point the fusion promoting effect was even more apparent (Fig. 4E). The fusogenic activity of Doc2b was SNARE-dependent (Fig. 4E). If Doc2b acts as a $Ca^{2+}$ sensor for spontaneous synaptic vesicle fusion it is likely to be activated by intracellular $Ca^{2+}$ in the sub-μM range (15). Consistent with this, Doc2b bound to membranes with a sub-μM affinity (fig. S8) and at sub-μM $Ca^{2+}$ Doc2b promoted fusion (Fig. 4F). The in vitro fusion-promoting effect of Doc2b far exceeded that of synaptotagmin-1 and Doc2a (Fig. 4G). Next we tested if $Ca^{2+}$-dependent membrane binding and SNARE binding were required for the strong fusion promoting effect of Doc2b. Indeed, both mutant $Doc2b^{4A}$ impaired in $Ca^{2+}$-dependent

phospholipid binding (fig. S8) and mutant Doc2b$^{K237, 319E}$ impaired in SNARE binding (Fig. 4A) were less effective (Fig. 4H).

## Ca$^{2+}$-dependence of Doc2b-driven spontaneous release

If Doc2b generates spontaneous events by acting as a Ca$^{2+}$ sensor, then mutagenesis of its Ca$^{2+}$-binding site should affect the Ca$^{2+}$-dependence of spontaneous release. We thus expressed wildtype or mutant Doc2b in DKO neurons in network cultures (10, 12). The Ca$^{2+}$-dependence of spontaneous release was assessed by superfusing neurons with increasing extracellular Ca$^{2+}$ concentrations (from 0.2 mM to 10 mM; Fig. 5A). As expected, the spontaneous release rate increased with increasing Ca$^{2+}$ concentrations (Fig. 5B) (12). Mutant Doc2b$^{D218,220N}$, which mimics a dominant active Ca$^{2+}$-bound state of the C2A domain (18), caused a massive increase in spontaneous release rate, especially at low Ca$^{2+}$-concentrations (Fig. 5B-C). The Ca$^{2+}$- dependence of spontaneous release was almost completely lost in Doc2b$^{D218,220N}$ expressing cells. This correlated well with the Ca$^{2+}$-independent binding of the isolated C2A$^{D218,220N}$ domain to liposomes (Fig. 5E and fig. S8). DKO neurons expressing wildtype Doc2b had a higher spontaneous release rate at each Ca$^{2+}$ concentration than DKO neurons expressing GFP (Fig. 5B). Even in cells lacking Doc2 we observed more spontaneous release events at higher Ca$^{2+}$ concentrations, albeit less pronounced than in wildtype or rescued cells. Thus, as seen before (Fig 1), additional high affinity Ca$^{2+}$-sensors for spontaneous release may exist. At high extracellular Ca$^{2+}$-concentrations (5-10 mM) the spontaneous release rate increased in all groups, a release which may be supported by synaptotagmins (12). We also expressed Doc2b$^{WT}$ and Doc2b$^{D218,220N}$ in neurons from wildtype mice. Consistent with the data from DKO neurons, expression of each construct increased the spontaneous release rate. This was most pronounced in Doc2b$^{D218,220N}$-expressing cells (Fig. 5C). Conversely, overexpression of a loss of function mutant, Doc2b$^{K237,319E}$, caused significantly lower mEPSC frequencies at all Ca$^{2+}$ concentrations compared to Doc2b$^{WT}$ in DKO neurons (Fig. 5D). Because PIP$_2$ binds to the same region as SNAREs (17, 20), we cannot exclude a contribution of PIP$_2$ binding in this experiment. However our in vitro fusion assay was performed in absence of PIP$_2$ so here, the profound loss of Doc2b$^{K237,319E}$ function was almost certainly due to its loss of Ca$^{2+}$-dependent SNARE-binding (Fig. 4). Thus, manipulation of the Ca$^{2+}$-dependence of Doc2b affected the Ca$^{2+}$-dependence of spontaneous release, implicating Doc2b as a Ca$^{2+}$ sensor.

## Conclusions

Here we have found that Doc2b is a high affinity Ca$^{2+}$-sensor for half (hippocampus) or most (cerebellum) of the spontaneous neurotransmitter release events in CNS synapses. Doc2b acts as a functional synaptotagmin-1 analogue operating at smaller Ca$^{2+}$ increases and with higher in vitro fusion efficiency. In contrast to synaptotagmin-1 that has the appropriate affinity to respond to high Ca$^{2+}$ peaks occurring at release sites, Doc2b requires less than 1 μM Ca$^{2+}$ to enhance fusion. Doc2b is not associated with the synaptic vesicle via a transmembrane domain like synaptotagmins and translocates to membranes in response to Ca$^{2+}$ (25), a quality that would explain why evoked release was unaffected in our study. Nevertheless, it is likely that Doc2 proteins are located not far from release sites due to its interactions with SNARE-complexes (Fig 4A), Munc13 (42) and Munc18 (17). Given the sensitivity of Doc2-dependent spontaneous release to BAPTA-chelation (Fig 1E) the protein must be activated close to a source of Ca$^{2+}$, whether this be a Ca$^{2+}$-channel or an intracellular Ca$^{2+}$-store (13-16).

Spontaneous release events appear to be mechanistically heterogeneous. In the synapses studied here, most spontaneous events are Ca$^{2+}$-dependent and require Doc2. Because the

spontaneous release in the absence of Doc2 remained sensitive to BAPTA and prolonged neuronal firing at 5 Hz, one or more additional high-affinity $Ca^{2+}$- sensors probably exist. Finally, BAPTA did not block all spontaneous release events and thus, some events are probably truly $Ca^{2+}$-independent, which may be supported by factors such as synaptotagmin-12 (11).

Because multiple C2 domain-containing proteins are co-expressed in synapses and at least Doc2b and synaptotagmin-1 compete for SNARE complex binding, a certain degree of redundancy probably exists among these protein families. The concentrations of various C2-domain proteins and their $Ca^{2+}$-affinity will determine the $Ca^{2+}$-sensitivity of release and the release probability of vesicles. Differences in this balance may explain the large differences in spontaneous release frequencies observed among neurons. Along the same lines, it is conceivable that the increased frequency of spontaneous release in synaptotagmin-1 deficient mice is triggered by Doc2 or other C2 domain-containing proteins (40, 43, 44) that replace synaptotagmin- 1 in its absence, where effectively synaptotagmin would appear to clamp fusion (12). Finally, while spontaneous vesicle fusion could conceivably have been dependent on SNARE assembly alone, we now show that many of these events require the action of curvature inducing multiple C2 domain proteins.

**One-sentence summary**

Doc2 proteins are functional analogs of synaptotagmins and act by membrane curvature induction to trigger spontaneous neurotransmitter release upon small $Ca^{2+}$ increases.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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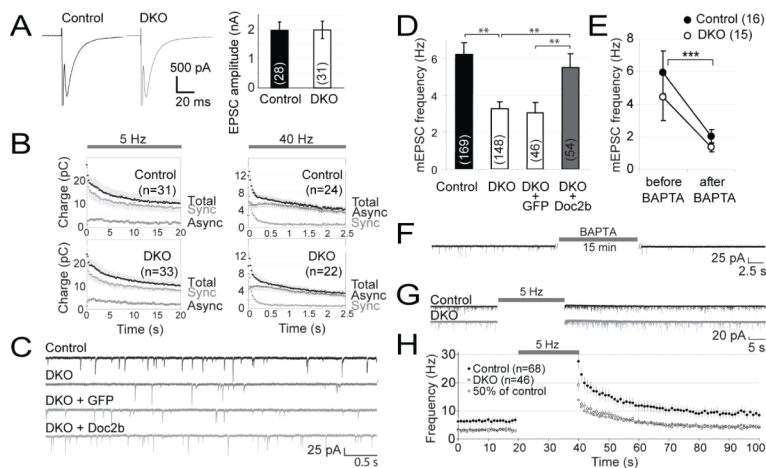
**Fig. 1.**
Impaired spontaneous neurotransmission in Doc2a/b-deficient mice. Hippocampal neurons were cultured on microislands to promote self-innervation. **(A)** Averaged evoked excitatory postsynaptic currents (EPSCs) in control and DKO neurons. **(B)** Evoked EPSC charge (mean ± sem) during repeated stimulation at 5 or 40 Hz. The synchronous and asynchronous component were estimated as previously reported (24). **(C)** Representative traces of spontaneous EPSCs in wild-type or DKO cells. To rescue the phenotype, GFP or Doc2b were acutely expressed in DKO cells. **(D)** Average spontaneous release frequency in control and DKO cells and rescue by Doc2b overexpression. Cell numbers are indicated between brackets. **, P<0.01 **(E)** Average mEPSC frequency before and after intracellular loading of the Ca$^{2+}$ chelator BAPTA. The average spontaneous release rate varied between experiments, but our experimental design prevents confounding effects thereof (fig. S2E) **(F)** Example trace representative for the data in (E), taken from a DKO cell before and after BAPTA loading. **(G)** Typical recordings before and after repetitive stimulation at 5 Hz. **(H)** Individual release events were binned in 1 s time intervals to monitor their average frequency immediately after repetitive stimulation. Squares were calculated as 50% of the frequency in control cells.
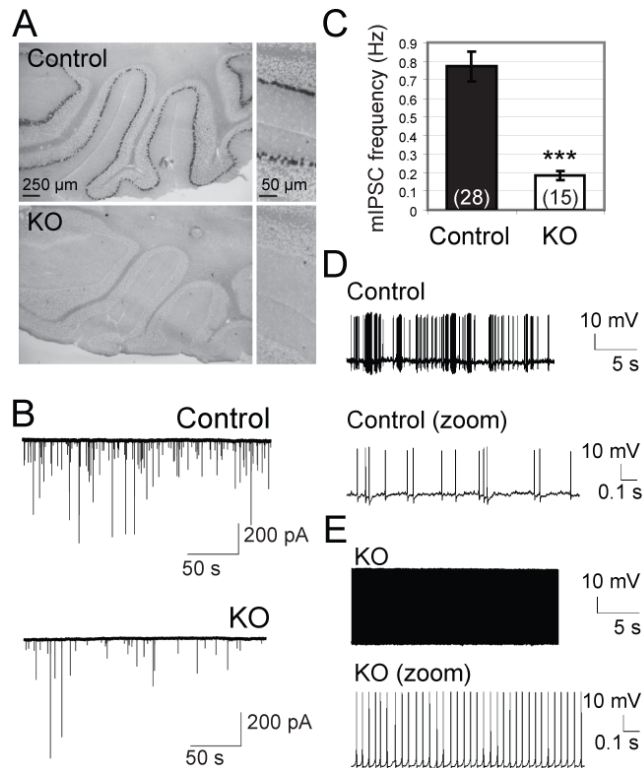
**Fig. 2.**
Reduced frequency of spontaneous events in Purkinje cells lacking Doc2b. **(A)** Doc2b mRNA was detected by in situ hybridization in cerebellar Purkinje cells from wildtype, but not *Doc2b*$^{-/-}$ (KO) mice. **(B)** Typical Voltage-clamp recordings in acute slices **(C)** Mean frequency of spontaneous inhibitory postsynaptic currents (mIPSCs) in KO mice and age-matched control littermates (n=15 and 28 cells; N=2 and 3 mice respectively). ***, p<0.0001. **(D-E)** Current clamp recordings of Purkinje cell firing patterns in controls (D, 8 cells from 3 mice) and KO cells (E, 7 cells from 2 mice).
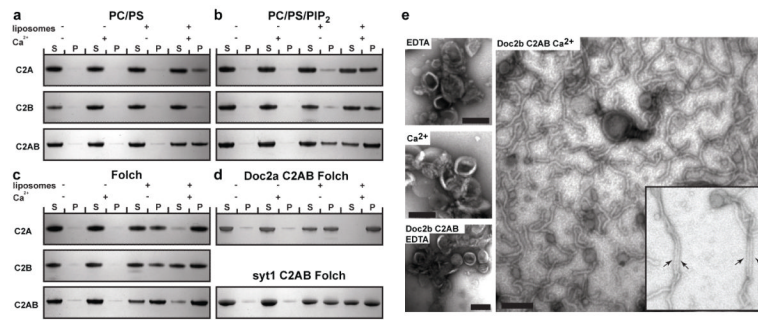
**Fig. 3.**
Membrane-binding and curvature induction by Doc2b. **(A-D)** Liposome cosedimentation assays to characterize the membrane binding properties of the Doc2b C2A, C2B, C2AB, Doc2a C2AB and synaptotagmin-1 (Syt1) C2AB domains. Liposomes composed of the indicated lipids were incubated with the Doc2b C2A, C2B or C2AB domains. Liposome-bound protein was co-sedimented by ultracentrifugation. 8% of the supernatant and pellet fraction were run on 4-12% gradient gels. Proteins were visualized by Coomassie staining. S indicates unbound protein in the supernatant while P indicates liposome-bound and thus co-pelleted protein. No or very little protein sedimented in the absence of liposomes. **(E)** $Ca^{2+}$-dependent tubulation of liposomes by the Doc2b C2AB domain. Folch liposomes were incubated in the absence or presence of Doc2b C2AB and/or $Ca^{2+}$ and processed for electron microscopy by negative stain. The arrows indicate bundles of closely aligned tubules. Scale bar 100 nm. All data shown are representatives of at least 3 independent experiments. (PS: phosphatidylserine, PC: phosphatidylcholine, $PIP_2$: phosphatidylinositol(4,5)bisphosphate)
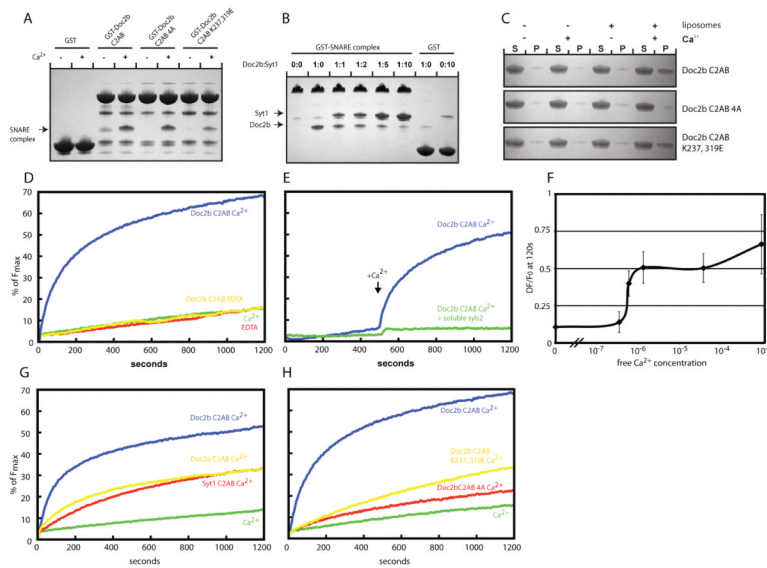
**Fig. 4.**
SNARE complex binding and promotion of SNARE-dependent membrane fusion by Doc2b.
**(A)** Coomassie stained gels showing a pull down experiment in the presence or absence of
$Ca^{2+}$ using the indicated Doc2b fusion proteins as bait and the purified SNARE complex as
prey. The co-pelleted SNARE complex is indicated by an arrow. **(B)** Coomassie stained gels
showing a pull down in the presence of $Ca^{2+}$ using the GST-SNARE complex as bait and
the Doc2b and synaptotagmin-1 C2AB domains as prey. GST served as control for
unspecific binding by the C2AB domains of Doc2B and synaptotagmin-1, respectively. The
co-pelleted Doc2B and synaptotagmin-1 C2AB domains are indicated by arrows. **(C)**
Liposome co-sedimentation assay in the presence or absence of $Ca^{2+}$ using the indicated
proteins and liposomes composed of 20% PS, 70% PC and 10% cholesterol. **(D)** In vitro
membrane fusion assay using reconstituted full length SNAREs in the absence or presence
of $Ca^{2+}$ and/or 7.5 μM Doc2b C2AB. **(E)** In vitro membrane fusion assay as in (D) but in
the presence or absence of the soluble SNARE domain of synaptobrevin. $Ca^{2+}$ was added at
500 s. **(F)** $Ca^{2+}$ dose-dependence curve showing the change of fluorescence at 120 s in the
reconstituted fusion assay in the presence of 7.5 μM Doc2b. Doc2b efficiently promotes
fusion at sub-μM $Ca^{2+}$ concentrations. The graph summarizes data from 3 independent
experiments. **(G)** Comparison of the fusion promoting activities of the Doc2b, Doc2a and
synaptotagmin-1 C2AB domains. **(H)** Fusion experiment using the indicated Doc2b C2AB
proteins reveals that $Ca^{2+}$-dependent membrane and SNARE-binding are required for
efficient fusion promotion. All data shown are representatives of at least 3 independent
experiments.
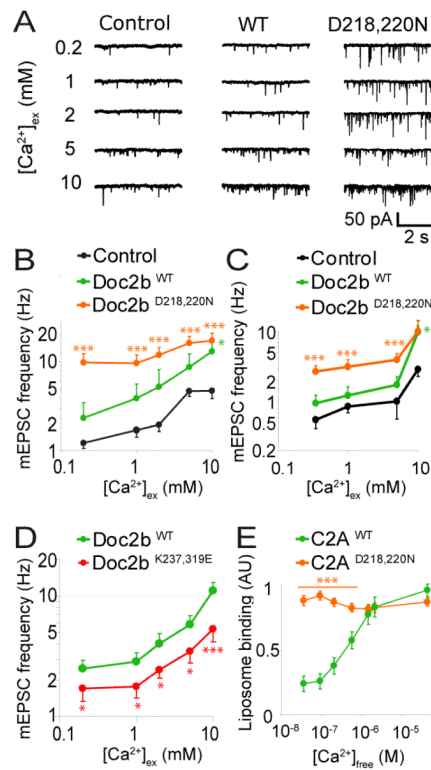
**Fig. 5.**
The $Ca^{2+}$-dependence of Doc2b determines that of spontaneous release in rescued DKO neurons. **(A)** Typical mEPSC recordings in DKO cells rescued with GFP (control), Doc2b$^{WT}$ or Doc2b$^{D218,220N}$. Spontaneous mEPSCs were recorded in network cultures of hippocampal neurons in the presence of TTX and gabazine. Increasing concentrations of extracellular $Ca^{2+}$ were administered to the same cell. **(B)** Mean mEPSC frequencies ± sem in DKO cells expressing Doc2b$^{WT}$ (n=10), Doc2b$^{D218,220N}$ (n=26) or GFP as a control (n=21) **(C)** Mean mEPSC frequencies of the same constructs expressed in wildtype cells. The average mEPSC frequency varied between experiments, but our experimental design prevents confounding effects thereof (see Fig S2E) **(D)** Mean mEPSC frequencies in DKO cells expressing Doc2b$^{WT}$ (n=35) or mutant Doc2b$^{K237,319E}$ (n=31) **(E)** $Ca^{2+}$-dependent liposome-binding by the isolated C2A domain of Doc2b$^{WT}$ and Doc2b$^{D218,220N}$ (n=6). *** (p<0.005); ** (p<0.01), * (p<0.05).