

Synaptotagmin 7 docks synaptic vesicles to support facilitation and Doc2 α -triggered asynchronous release

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Zhenyong Wu, Grant F. Kusick, Manon M. M. Berns, Sumana Raychaudhuri, Kie Itoh, Alexander M. Walter, Edwin R. Chapman , Shigeki Watanabe 

Department of Neuroscience, University of Wisconsin-Madison, Madison, USA • Howard Hughes Medical Institute, Madison, USA • Department of Cell Biology, Johns Hopkins University, School of Medicine, Baltimore, MD, USA • Biochemistry, Cellular and Molecular Biology Graduate Program, Johns Hopkins University, School of Medicine, Baltimore, MD, USA • Department of Neuroscience, University of Copenhagen, Copenhagen, Denmark • Molecular and Theoretical Neuroscience, Leibniz-Institut für Molekulare Pharmakologie, FMP im CharitéCrossOver, Berlin, Germany • Solomon H. Snyder Department of Neuroscience, Johns Hopkins University, School of Medicine, Baltimore, MD, USA

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Abstract

Despite decades of intense study, the molecular basis of asynchronous neurotransmitter release remains enigmatic. Synaptotagmin (syt) 7 and Doc2 have both been proposed as Ca²⁺ sensors that trigger this mode of exocytosis, but conflicting findings have led to controversy. Here, we demonstrate that at excitatory mouse hippocampal synapses, Doc2 α is the major Ca²⁺ sensor for asynchronous release, while syt7 supports this process through activity-dependent docking of synaptic vesicles. In synapses lacking Doc2 α , asynchronous release after single action potentials is strongly reduced, while deleting syt7 has no effect. However, in the absence of syt7, docked vesicles cannot be replenished on millisecond timescales. Consequently, both synchronous and asynchronous release depress from the second pulse onward during repetitive activity. By contrast, synapses lacking Doc2 α have normal activity-dependent docking, but continue to exhibit decreased asynchronous release after multiple stimuli. Moreover, disruption of both Ca²⁺ sensors is non-additive. These findings result in a new model whereby syt7 drives activity-dependent docking, thus providing synaptic vesicles for synchronous (syt1) and asynchronous (Doc2 and other unidentified sensors) release during ongoing transmission.

eLife assessment

In this **important** study, the authors identify distinct roles for the calcium sensors Synaptotagmin 7 and Doc2 α in the regulation of asynchronous release and calcium-dependent synaptic vesicle docking in hippocampal neurons. The current work adds to the field by placing the role of the two proteins in a new context, where Synaptotagmin 7 acts to promote synaptic vesicle docking and capture after a stimulus, while Doc2 α has a role in specifically driving the asynchronous component of release as a calcium sensor. The methods, data, and analyses provide **convincing** support for the conclusions.

Introduction

Ca^{2+} -triggered neurotransmitter release involves at least two kinetically distinct components: one is synchronous, and sets in after tens of microseconds (Sabatini and Regehr, 1996) of a stimulus and completes within several milliseconds. The other component is asynchronous; it sets in more slowly and can persist for tens or hundreds of milliseconds (Goda and Stevens, 1994; Kaeser and Regehr, 2014). The molecular basis of synchronous release has been characterized in depth, and there is broad agreement that the underlying Ca^{2+} sensors are two members of the synaptotagmin (syt) family of proteins, isoforms 1 and 2 (Stevens and Sullivan, 2003). The Ca^{2+} sensors that regulate asynchronous release (AR), however, remain a subject of controversy.

AR is thought to play a critical role in information processing and circuit function (Iremonger and Bains, 2007; Kaeser and Regehr, 2014; Manseau et al., 2010) and has been implicated in epileptiform activity in rats and humans (Jiang et al., 2012). However, it is impossible to directly test how slow transmission impacts neural networks without knowing what proteins are specifically required for AR.

Two classes of proteins have been proposed as Ca^{2+} sensors for AR in vertebrates: Doc2 and syt7. Two isoforms of Doc2, α and β function as Ca^{2+} sensors; α mediates AR in hippocampal neurons, and loss of AR in α knockout (KO) neurons can be rescued by β (Yao et al., 2011). Both Doc2 and syt7 exhibit the slow Ca^{2+} -regulated membrane-binding kinetics and high affinity required for AR (Hui et al., 2005; Xue et al., 2015; Yao et al., 2011). In cultured hippocampal neurons, we previously found that Doc2 α drives significant levels of AR both after single action potentials and during trains (Gaffaney et al., 2014; Xue et al., 2018, 2015; Yao et al., 2011). This activity depends on Ca^{2+} binding (Xue et al., 2018, 2015). However, no significant changes in AR were observed in Purkinje cell to deep cerebellar nuclei synapses (Khan and Regehr, 2020) and in autaptic cultured hippocampal neurons lacking Doc2 (Groffen et al., 2010). These varying results have left the importance of Doc2 for AR in doubt.

Syt7 has received more attention (Huson and Regehr, 2020), but its exact role in AR is still uncertain. Syt7 is important for AR at the zebrafish neuromuscular junction (Wen et al., 2010). However, conflicting evidence has accumulated for mammalian central synapses. In syt7 KO mouse cortical neurons, no synaptic phenotypes were initially observed (Maximov et al., 2008). Later studies found defects in AR when deleting syt7 or preventing it from binding Ca^{2+} in cultured hippocampal neurons (Bacaj et al., 2013), in the cerebellum at inhibitory synapses formed between basket cells and Purkinje neurons as well as at granule cell synapses (Chen et al., 2017; Turecek and Regehr, 2018), and at excitatory synapses between pyramidal cells and Martinotti neurons in the neocortex (Deng et al., 2020). However, unlike Doc2 α , the role of syt7 in AR is usually only apparent when more than one stimulus is applied. Among these studies, deleting syt7 decreased AR—after a single action potential—only in granule cell synapses (Turecek and Regehr, 2018) and in syt1 KO cultured hippocampal neurons (Bacaj et al., 2013). In inhibitory synapses of the inferior olive, where release after single action potentials is often highly asynchronous, deleting syt7 actually makes release even slower (Turecek and Regehr, 2019). Thus, the importance of syt7 and Doc2 α in AR may depend, at least in part, on the frequency of stimulation, the number of stimuli applied, and the synapse under study. The reason for these differences is unknown, reflecting a lack of mechanistic insight into how these proteins support AR.

To directly compare the roles of Doc2 α and syt7 in AR, we performed glutamate sensor imaging and electrophysiology experiments in single and double knockout mouse hippocampal neurons. We then explored how these sensors act on synaptic vesicles using zap-and-freeze electron microscopy and mathematical modeling. In both cultured neurons and acute slices, AR after single

action potentials was reduced in Doc2 α KOs, but not in syt7 KOs, suggesting that Doc2 α more directly contributes to AR. In contrast, AR during train stimulation was reduced to a similar extent in both syt7 and Doc2 α KO neurons. Ultrastructural analysis revealed that, in syt7 KO neurons, docked synaptic vesicles fail to recover on millisecond timescales (Kusick et al., 2020). Consequently, both synchronous and asynchronous release quickly and profoundly depress during train stimulation. By contrast, in Doc2 α KO neurons this syt7-dependent ultrafast recruitment of vesicles was unaffected. Thus, with 50-60% less AR, more newly-recruited vesicles are available for synchronous release, leading to slower depression in Doc2 α KO neurons. AR in Doc2 α /syt7 DKO neurons was similar to Doc2 α KO, indicating that the remaining AR is triggered by one or more as-yet-unidentified proteins. Our findings are consistent with a sequential model wherein synaptic vesicle docking, but not fusion, is facilitated by syt7 during activity. Syt1, Doc2 α , and additional sensor(s) act downstream and compete with each other to trigger exocytosis. Overall, we propose that during repetitive stimulation, syt7 supplies docked vesicles for synchronous release and Doc2 α -triggered AR.

Results

Doc2 α , but not syt7, triggers asynchronous release in response to single action potentials

To directly monitor glutamate release, we transduced an optical glutamate reporter (iGluSnFR) into cultured mouse hippocampal neurons (Marvin et al., 2018, 2013; Fig. 1A,B and Fig. S1). This approach bypasses postsynaptic factors that influence the time course of transmission as monitored using traditional patch-clamp recordings and allows individual AR events to be measured directly (Vevea et al., 2021). We applied single stimuli and monitored the timing of glutamate release at the single-bouton level (Fig. 1C) from Doc2 α knockout (KO, *Doc2 α em1Smoc/em1Smoc*; Sakaguchi et al., 1999) and syt7 KO (*Syt7^{tm1Nan/tm1Nan}*; Chakrabarti et al., 2003) neurons and their respective wild-type littermates (WT – *Doc2 α ^{+/+}* or *Syt7^{+/+}*). We used a medium affinity iGluSnFR (A184V; $K_D = 40 \mu\text{M}$) for these single-stimulus experiments (Marvin et al., 2018). Due to the fast activation kinetics of this sensor, compared to its relatively slow relaxation rate (Marvin et al., 2018; Vevea and Chapman, 2020; Fig. S1B), the timing of each release event was quantified by measuring the time-to-peak of each response. These data were plotted as histograms using 10 ms bins (Fig. 1A,B and Fig. S1A). Peaks detected at the 10 ms time point were defined as the synchronous component of release, and all later peaks were defined as AR events (Vevea and Chapman, 2020).

In WT neurons, 64% of release events occurred at the first 10 ms time point (synchronous release: $n = 1000$ release events); the rest of the events (36%) were defined as asynchronous (Fig. 1C). This ratio of synchronous to asynchronous release is likely an underestimate, since our analysis only counts the number of peaks ('events') and does not take into account multiquantal peaks resulting from near-simultaneous multivesicular release. We have previously determined by quantal analysis that most synchronous peaks after a single action potential are multiquantal, while for AR there are still multiquantal events but they are in the minority (Vevea et al., 2021). So in these data, the total amount of synchronous release is underestimated more so than AR; sophisticated quantal analysis using the A184V iGluSnFR recently found the percentage of total release that is AR to be ~25%, with otherwise similar results to ours (Mendonça et al., 2022). Nonetheless, this approach faithfully distinguishes synchronous from asynchronous release, as validated by analyzing release from WT neurons treated with EGTA-AM, which reduces AR by chelating residual Ca^{2+} (Chen and Regehr, 1999). This treatment greatly reduced AR (Fig. 1C; 88% synchronous and 12% asynchronous). Syt7 KO neurons had no apparent phenotype (Fig. 1C, D; 66% synchronous and 34% asynchronous; $p = 0.39$). The 2% reduction in AR in the KO was not statistically significant here. However, we note that this small effect is consistent with our earlier measurements using iGluSnFR, which detected a very small but statistically significant

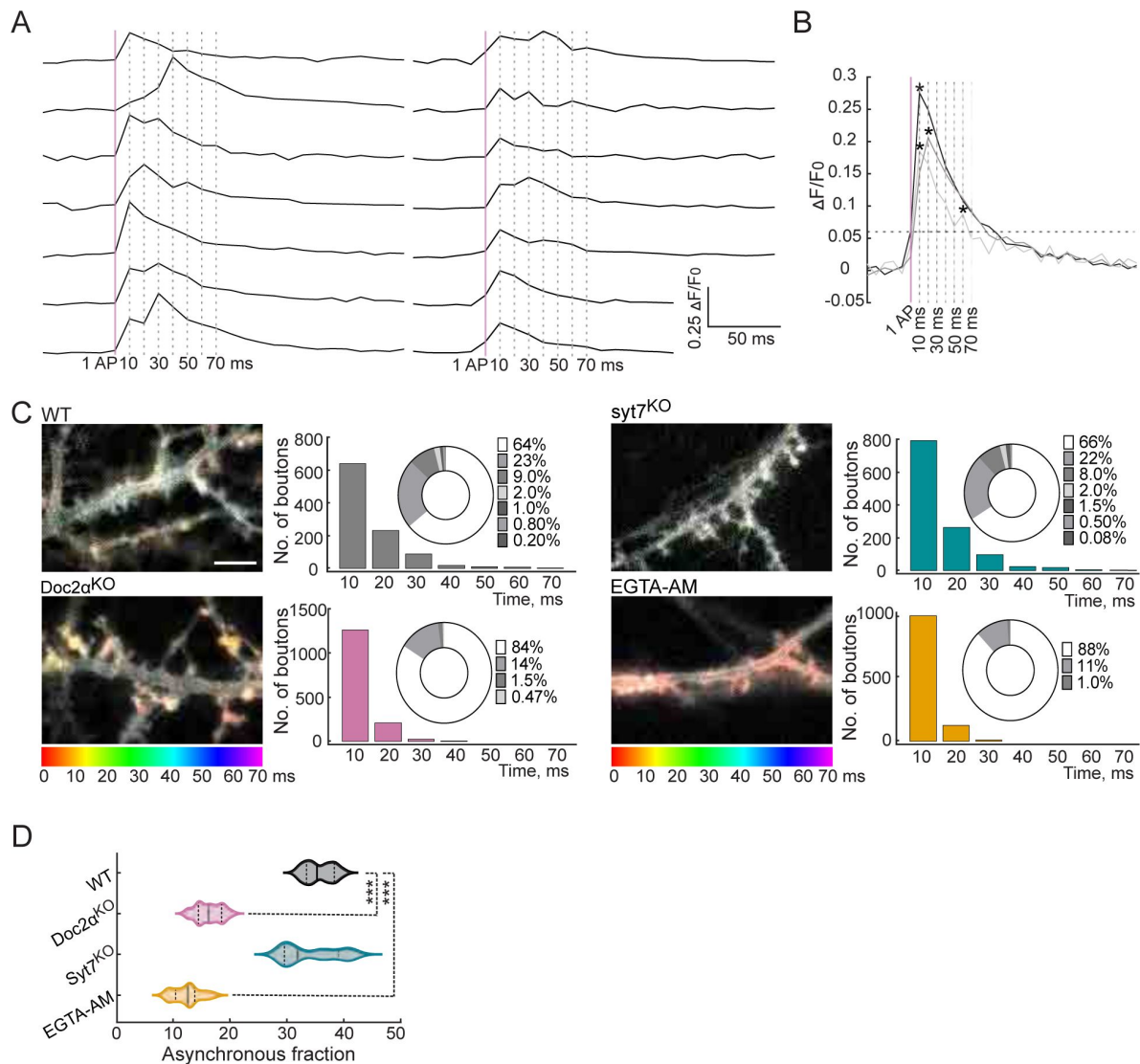


Fig. 1.

Doc2 α , and not syt7, drives most of the asynchronous glutamate release triggered by a single action potential in cultured hippocampal neurons.

(A) Raw iGluSnFR (A184V) traces from cultured WT mouse hippocampal neurons. A single AP was applied, and images were captured every 10 ms. Peaks that appeared at the first 10 ms timepoint were defined as synchronous release events; peaks that appeared at later time points were defined as asynchronous release events. The vertical magenta line indicates the stimulus, and the vertical dashed lines indicate the imaging frames at 10, 20, 30, 40, 50, 60, and 70 ms.

(B) Three representative traces illustrate synchronous and AR peaks. The horizontal dashed line is five-times the standard deviation of the baseline noise. Individual peaks are indicated by asterisks. Traces showing single synchronous (black) and asynchronous peaks (gray), along with a trace in which both kinds of events occurred (light gray), are shown.

(C) Color coding and binning the temporal changes in iGluSnFR signals within 70 ms after a single stimulus for WT ($n=15$ fields of view/coverlips; from 4 independent litters), Doc2 α ^{KO} ($n=17$; from 5 independent litters), syt7^{KO} ($n=15$; from 4 independent litters), and EGTA-AM-treated neurons ($n=18$; from 4 independent litters). The temporal color code is red (initiation of stimulus) to purple (70 ms after the stimulus); scale bar, 5 μ m. Histograms of iGluSnFR ($\Delta F/F_0$) peaks were generated using 10 ms binning. Samples were color-coded as follows: WT (gray), Doc2 α ^{KO} (reddish), syt7^{KO} (cyan), and EGTA-AM (orange) treated neurons. The histograms include donut graph insets to display the fraction of release in each bin.

(D) Violin plot showing the % AR (release after 10 ms) for each condition. One-way ANOVA, followed by Dunnett's test, was used to compare mutants against WT. *** indicates $p<0.001$.

decrease in asynchronous release in syt7 KO neurons (Vevea et al., 2021 [DOI](#)). By contrast, the asynchronous component was markedly reduced in Doc2α KO neurons (**Fig. 1B,C** [DOI](#); 84% synchronous and 16% asynchronous). The peak average fluorescence intensity in both mutants was similar to WT (**Fig. S1C** [DOI](#)), demonstrating that synchronous release is not affected in these mutants (Vevea et al., 2021 [DOI](#); Yao et al., 2011 [DOI](#)). These findings indicate that Doc2α, but not syt7, plays an important role in AR in response to a single stimulus.

We next turned to acute hippocampal slices to assay Doc2α and syt7 function in a more native circuit. We applied a single stimulus to Schaffer collaterals and measured evoked excitatory postsynaptic currents (EPSCs) from CA1 neurons by whole-cell patch clamp (**Fig. 2A** [DOI](#), and **S4** [DOI](#)). As with iGluSnFR imaging in cultured neurons (**Fig. 1** [DOI](#)), syt7 KO showed no phenotype – the amplitude and kinetics of EPSCs were nearly identical to those of WT (**Fig. 2B-D** [DOI](#)). By contrast, in Doc2α KO, the EPSC amplitude was unchanged, but the total charge transfer was reduced by 35% (**Fig. 2B-D** [DOI](#)). Cumulative charge transfer data were fitted with double exponential functions, reflecting two kinetic components of release (**Fig. 2C** [DOI](#); Goda and Stevens, 1994 [DOI](#)). The fast component, representing synchronous release, was similar across all three genotypes. However, the slow, asynchronous component was decreased by 61% in Doc2α KO neurons ($p < 0.0001$), and by 10% in syt7 KO neurons (**Fig. 2D** [DOI](#); $p = 0.15$), though this latter difference was not statistically significant. EGTA-AM treatment of WT slices reduced the slow component by 74% (**Fig. 2B-D** [DOI](#); $p < 0.0001$). Finally, the time constants for both components of evoked EPSCs were similar across all genotypes (**Fig. 2D** [DOI](#)). Together, these data further indicated that Doc2α, but not syt7, is required for a large fraction of AR after single action potentials in excitatory mouse hippocampal synapses.

Both Doc2α and syt7 are important for asynchronous release after repeated action potentials

We then addressed the roles of Doc2α and syt7 in AR during train stimulation. Fifty stimuli, at 10 and 20 Hz, were applied to cultured hippocampal neurons that expressed iGluSnFR (**Fig. 3A,B** [DOI](#)). To temporally resolve the response from each pulse during the train, we used a faster, lower-affinity version of iGluSnFR (S72A; $K_D = 200 \mu\text{M}$; Marvin et al., 2018 [DOI](#), 2013 [DOI](#)). These faster kinetics were essential to resolve individual peaks during train stimulation; however, this sensor underestimates the fraction of AR (~10% of total release for a single action potential) as compared to the A184V variant used above that overestimates the fraction of AR (~35% of total release for a single action potential). This is because S72A is less sensitive and misses many unquantal events; as discussed above, our analysis quantifies release by number of peaks, and most synchronous peaks after a single action potential are multiquantal, while most AR peaks are unquantal (Vevea et al., 2021 [DOI](#)). Still, the S72A variant reported the same phenotypes as the A184V variant after the first action potential (**Fig. 3B, C** [DOI](#)).

At both 10 and 20 Hz, WT neurons exhibited slight facilitation of synchronous release (measured as the peak average fluorescence intensity) in response to the second pulse, and thereafter depressed as the train went on, eventually reaching a steady state (**Fig. 3B, D** [DOI](#), and **S2A** [DOI](#)). In addition, the asynchronous fraction gradually increased during the train at both stimulation frequencies (**Fig. 3C** [DOI](#) and **S2B** [DOI](#)). We examined the first two responses in the train and found that paired pulse facilitation was similar between WT and Doc2α KO neurons (WT: 1.04 ± 0.06 , Doc2α KO: 1.03 ± 0.04 , $p = 0.9953$, with an interstimulus interval (Δt) = 100 ms; WT: 1.31 ± 0.08 , Doc2α KO: 1.28 ± 0.08 , $p > 0.9191$, $\Delta t = 50$ ms; **Fig. S2B** [DOI](#)). However, in KO neurons, synaptic depression set in more slowly from the third action potential onward, eventually reaching the same steady-state depression as WT later in the train. This is consistent with the idea that the sensors for synchronous and asynchronous release compete with each other, so that reducing AR promotes sustained synchronous release (Otsu et al., 2004 [DOI](#); Yao et al., 2011 [DOI](#)). In contrast, syt7 KO neurons displayed paired pulse depression instead of facilitation (syt7 KO: 0.57 ± 0.03 , $p < 0.0001$, $\Delta t = 1001$ ms; syt7 KO: 0.52 ± 0.03 , $p < 0.0001$, $\Delta t = 50$ ms; **Fig. S2B** [DOI](#)), as well as faster and

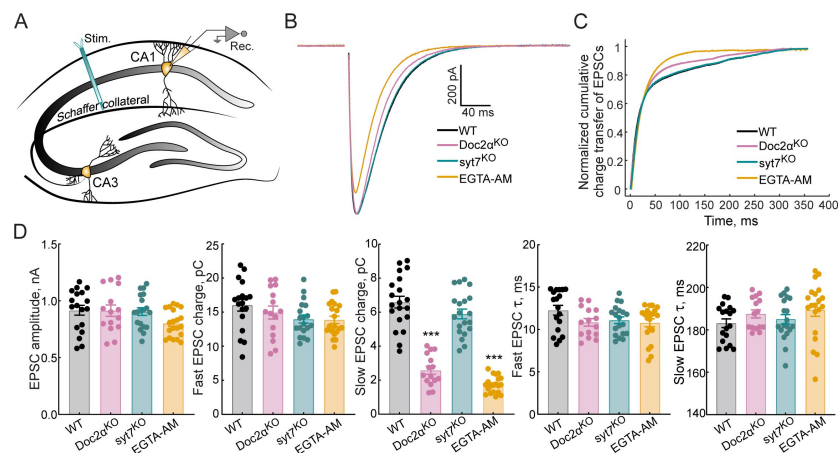


Fig. 2.

Doc2α, and not syt7, drives most of the AR triggered by a single action potential at Schaffer collaterals.

(A) Schematic of the recording scheme of hippocampal slices showing stimulation of the Schaffer collateral fibers and whole-cell patch clamp recordings of CA1 pyramidal neurons.

(B) Averaged traces of evoked EPSCs recorded from WT (n=18 recordings; from 5 independent litters), Doc2α^{KO} (n=15; from 4 independent litters), syt7^{KO} (n=19; from 6 independent litters), and EGTA-AM treated neurons (n=20; from 4 independent litters).

(C) Normalized cumulative EPSC charge transfer over 400 ms.

(D) Bar graphs, from corresponding groups in panels (B-C), summarizing the amplitude, fast and slow EPSC charge, and the fast and slow EPSC τ values. One-way ANOVA, followed by Dunnett's test, was used to compare mutants against WT. **, and *** indicate p < 0.01, and p < 0.001.

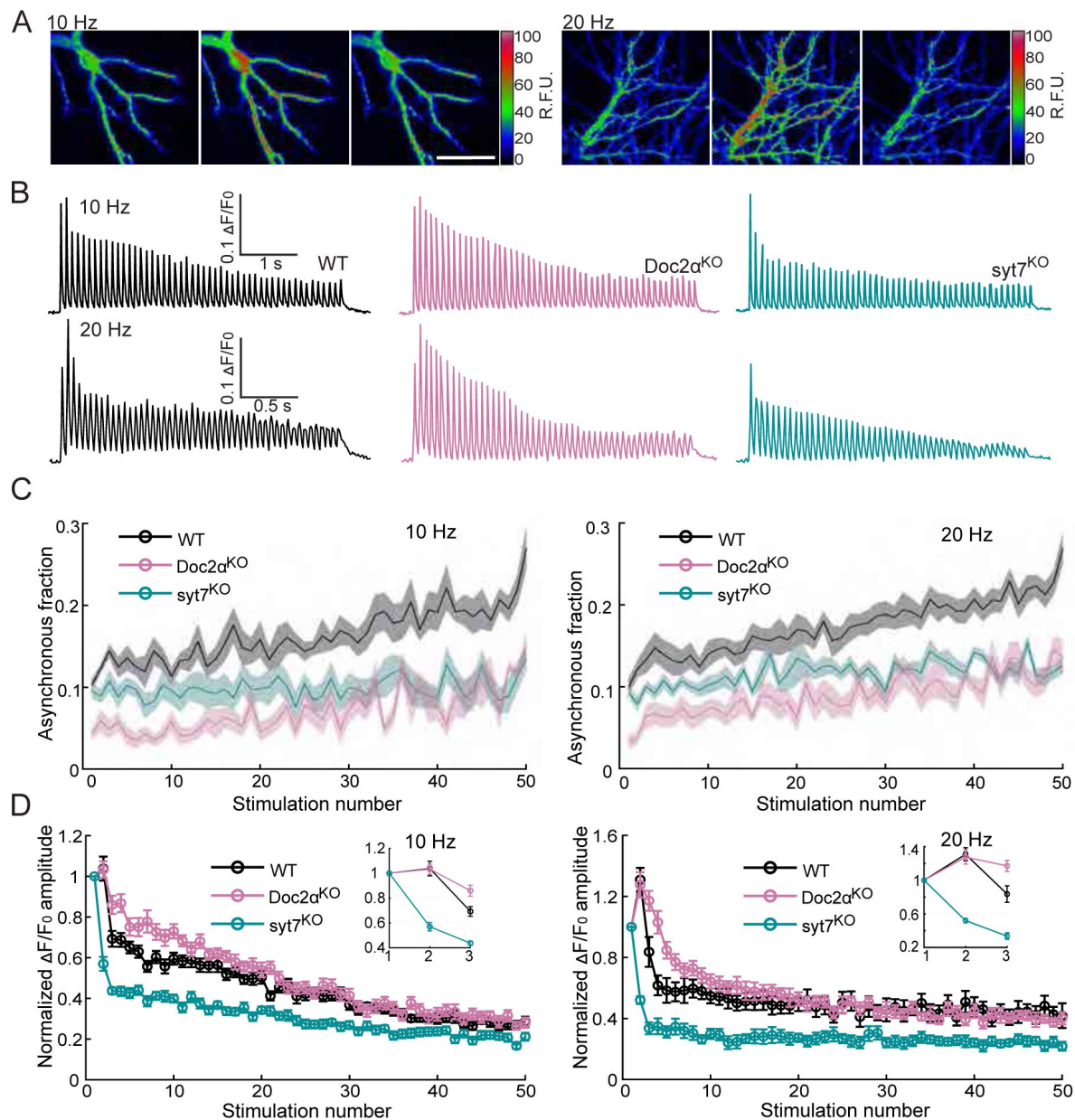


Fig. 3.

Both *Doc2α* and *syt7* contribute to asynchronous glutamate release during train stimulation but have opposite effects on short-term plasticity in cultured hippocampal neurons.

(A) Representative iGluSnFR images from trains of 50 APs at 10 Hz (left) or 20 Hz (right). Images on the left, middle and right each show a sum of 20 frames (200 msec) before, during, and after stimulation, respectively; scale bar, 40 μ m.

(B) Representative iGluSnFR responses from WT (10 Hz: $n=12$ fields of view/coverslips; from 3 independent litters; 20 Hz: $n=17$; from 4 independent litters), *Doc2α*^{KO} ($n=14$; from 4 independent litters; 20 Hz: $n=16$; from 4 independent litters), and *syt7*^{KO} neurons ($n=15$; from 3 independent litters; 20 Hz: $n=12$; from 3 independent litters), stimulated at 10 Hz (top) and 20 Hz (bottom).

(C) The AR fraction was defined as iGluSnFR peaks that occurred after the first 10 ms time point (i.e., from the 20 msec time point onward), divided by the total number of peaks that occurred at all time points between each stimulus. These data are plotted as a function of the stimulus number. Mean values (bold lines) $\pm$ SEM (shaded regions) are indicated.

(D) Plot of the normalized amplitude of iGluSnFR signals evoked by 50 stimuli at 10 and 20 Hz. Insets show the first three points of each trace on expanded scales.

more severe synaptic depression as the train progressed (**Fig. 3D**), consistent with previous reports (Chen et al., 2017; Jackman et al., 2016; Liu et al., 2014; Vevea et al., 2021). As in the single-stimulus measurements (**Fig. 1**), AR was decreased in Doc2a KO, but not syt7 KO, in response to the first pulse in the stimulus train. However, from the second action potential onwards, both KOs yielded reductions in AR as compared to WT, such that by the end of the train the fraction of AR was reduced by 53% and 50% in syt7 and Doc2a KO neurons, respectively (**Fig. 3A-C**; see also (Vevea et al., 2021)).

One potential source of the observed phenotype in syt7 KO during the train is variability among synapses. That is, the boutons that respond to the second pulse onward may have different release properties. To test such a possibility, we analyzed the distribution of the iGluSnFR signals from 923 boutons. We did not observe spatial segregation of AR during the first two responses (see **Fig. S3** for further analysis and details). Thus, the differences in AR between the first and second responses are not due to differences regarding which boutons responded during the first and second stimuli. Together, these data suggest that both Doc2a and syt7 contribute to AR from the second pulse onward during the train.

Next, we extended the train stimulation experiments to hippocampal slices, via patch clamp recordings (**Fig. 4A**), and observed the same trends that were obtained using iGluSnFR in cultured neurons (**Fig. 3**). Namely, peak amplitudes (synchronous release) depressed more slowly in Doc2a KO, and syt7 KO neurons had faster and more profound synaptic depression during the train, as compared to WT (**Fig. 4B**). Interestingly, loss of syt7 caused more severe synaptic depression than EGTA-AM. To estimate the extent of AR in these electrophysiological experiments, we calculated the tonic charge transfer, as previously described (Otsu et al., 2004; Yao et al., 2011), using the 20 Hz stimulation data. This approach revealed that syt7 and Doc2a KO neurons both exhibited 65% reductions in the tonic charge transfer component, similar to the EGTA-AM treatment condition (74%) (**Fig. 4C**). These data are all consistent with both Ca^{2+} binding proteins, Doc2a and syt7, playing important roles in AR driven by residual Ca^{2+} (Wen et al., 2010; Yao et al., 2011).

Doc2a mediates asynchronous fusion, while syt7 docks vesicles

Why is Doc2a important for AR after single and multiple stimuli, while syt7 only contributes starting with a second action potential? And why is short-term plasticity so strongly disrupted in syt7 KOs? Recent studies have proposed that synaptic vesicles rapidly transition from undocked to docked states during activity, and that this could, in principle, provide a common basis for AR, synaptic facilitation, and resistance to synaptic depression (Chang et al., 2018; Kobbersmed et al., 2020; Kusick et al., 2022; Miki et al., 2018, 2016; Neher and Brose, 2018; Silva et al., 2021). In this model, after Ca^{2+} influx, initially undocked and release-incompetent vesicles can dock and fuse ('two-step' release: Blanchard et al., 2020; Miki et al., 2018), resulting in AR, or dock and then fuse after another action potential, leading to facilitation and resistance to depression (Miki et al., 2016; Neher and Brose, 2018). Consistent with this notion, we previously found that a substantial fraction of vesicles undock in response to an action potential, and enter into a dynamic state in which they re-dock, within 15 ms, to replenish vacated release sites. We termed this process 'transient docking', since vesicles again undock over the next 100 ms (Kusick et al., 2020). Importantly, like AR and facilitation, transient docking was blocked by EGTA-AM. Thus, our imaging and electrophysiology findings could be explained by these two Ca^{2+} sensors playing distinct roles in docking and fusion, with Doc2a triggering AR, while syt7 mediates transient docking.

To test this hypothesis, we performed zap-and-freeze electron microscopy experiments (Kusick et al., 2020). This approach allows us to measure synchronous and asynchronous vesicle fusion, as well as depletion and replenishment of docked vesicles, in the same experiment. Vesicles that appear to be in contact with the plasma membrane are considered docked. Unstimulated syt7 and

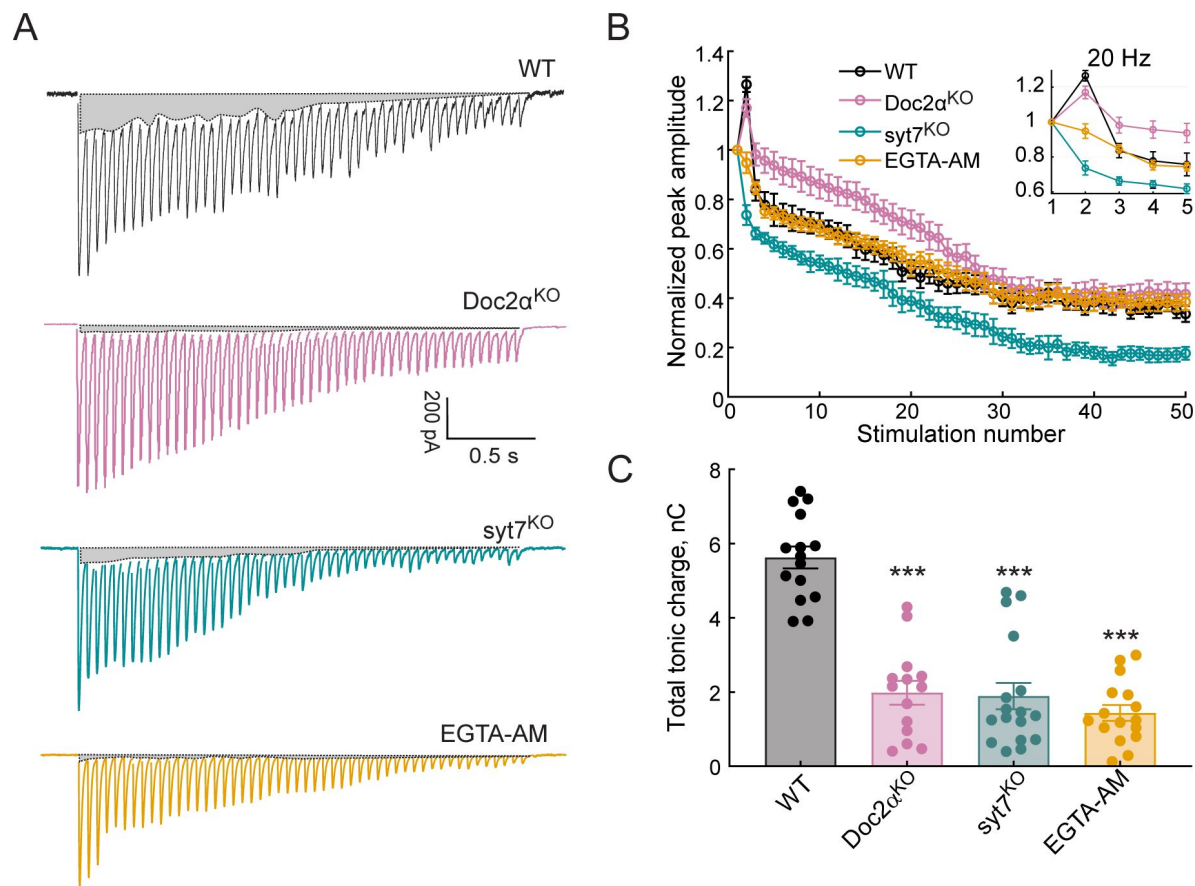


Fig. 4.

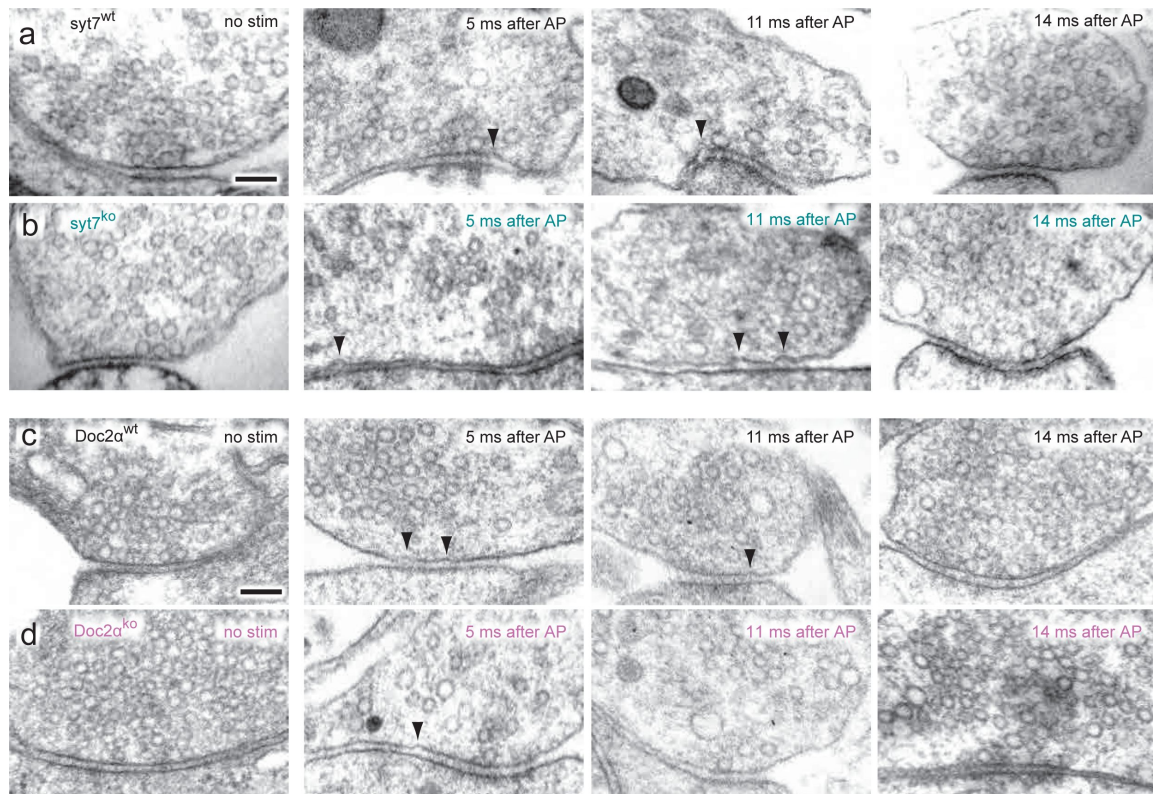
Both Doc2α and syt7 contribute to AR during train stimulation but have opposite effects on short-term plasticity at Schaffer collaterals.

(A) Representative EPSCs triggered by 20 Hz stimulus trains using WT, Doc2α^{KO}, syt7^{KO}, and EGTA-AM treated neurons. The shaded gray areas indicate the tonic charge component that likely corresponds to AR.

(B) The peak amplitude of each EPSC during the train was normalized to the first EPSC from WT (n=12 recordings; from 4 independent litters), Doc2α^{KO} (n=11; from 3 independent litters), syt7^{KO} (n=10; from 3 independent litters), and EGTA-AM treated neurons (n=11, from 3 independent litters).

(C) Bar graph showing the total tonic charge transfer (indicated by the grey areas in panel (A)), as an index of AR, from WT, Doc2α^{KO}, syt7^{KO}, and EGTA-AM treated hippocampal slices.

Doc2 α KO synapses appeared qualitatively normal (see **Fig. 5A-D** [↗](#), **6A-D** [↗](#), and **S5** [↗](#) for example micrographs). Knocking out either protein did not affect the number of docked vesicles at rest (**Fig. 6D-E** [↗](#)) nor the number and distribution of synaptic vesicles within 100 nm of the plasma membrane at the active zone (**Fig. S6D** [↗](#)). Therefore, the physiological defects in syt7 and Doc2 α KO synapses cannot be accounted for by any ultrastructural phenotypes at rest.



exocytic pits

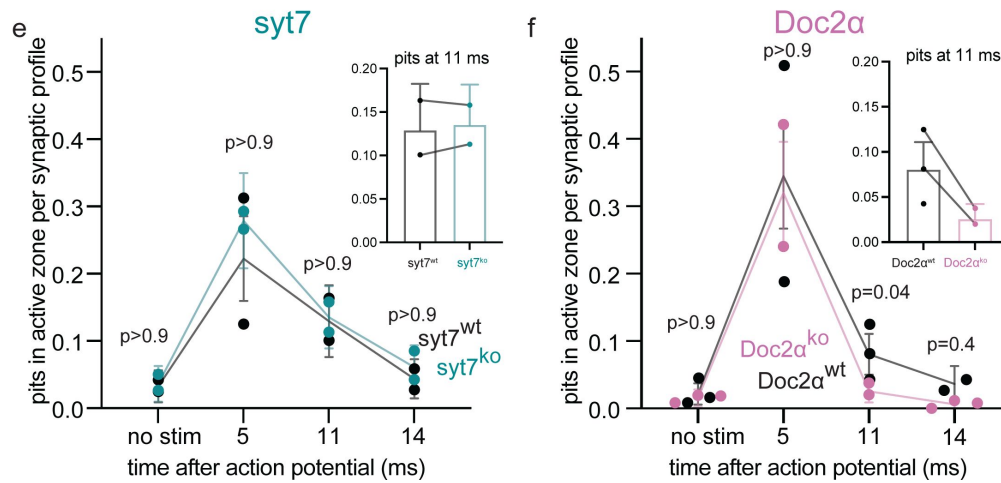


Fig. 5.

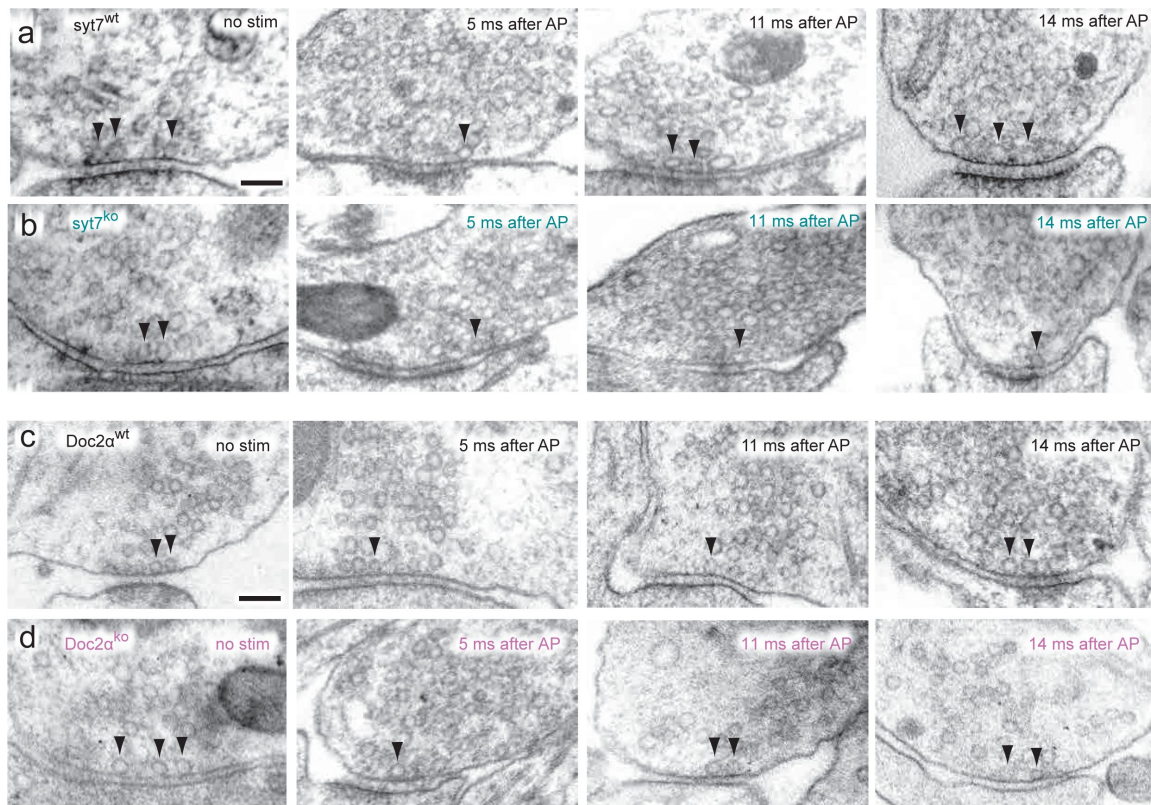
Doc2 α , but not syt7, is required for asynchronous synaptic vesicle fusion triggered by a single action potential in cultured hippocampal neurons.

(A-D) Example transmission electron micrographs of syt7^{WT} (A), syt7^{KO} (B), Doc2 α ^{WT} (C), and Doc2 α ^{KO} (D) synapses frozen at the indicated time points after an action potential (AP) or without stimulation (no stim). Arrows indicate pits in the active zone, which are presumed to be synaptic vesicles that have fused with the plasma membrane.

The number of pits in the active zone per synaptic profile (part of the synapse captured in a 2D section) in syt7 wild-type littermate controls and knockouts. Error bars indicate 95% confidence interval of the mean for all data pooled together; each dot indicates the mean from a single biological replicate. Inset: same data from the 11 ms time point from each genotype, with lesser y axis range; lines indicate data are from the same biological replicate (experiment), *not* that they are paired. *P* values are from comparisons between wild-type controls (nostim, *n* = 217; 5 ms, *n* = 216; 11 ms, *n* = 217; 14 ms, *n* = 230 synaptic profiles) and knockouts (no stim, *n* = 193; 5 ms, *n* = 215; 11 ms, *n* = 230; 14 ms, *n* = 212 synaptic profiles) frozen at the same time point.

(E) same as E, but for Doc2 α wild-type controls (no stim, *n* = 327; 5 ms, *n* = 229; 11 ms, *n* = 336; 14 ms, *n* = 192 synaptic profiles) and knockouts (no stim, *n* = 330; 5 ms, *n* = 231; 11 ms, *n* = 352; 14 ms, *n* = 321 synaptic profiles).

Number of biological replicates (from separate cultures frozen on different days and analyzed in separate randomized batches): 2 for each of the syt7 time points, 2 for 5 ms and 14 ms Doc2 WT, 2 for 5 ms and 11 ms Doc2 KO, 3 for Doc2 WT no stim and 11 ms, 3 for Doc2 KO no stim and 14 ms. The numbers of pits between wild type and knockout were compared using Brown-Forsythe ANOVAs with post-hoc Games-Howell's multiple comparisons tests. In pairwise tests, the same time point for wild type vs. knockout as well as each time point within each genotype were compared against each other (only comparisons between the same time point for wild type vs. knockout are shown, see Supplementary Table 1 for all pairwise comparisons). See [Fig. S6A](#) for active zone sizes from each condition and [Fig. S6B](#) for pit data normalized to the size of active zones.



docked vesicles

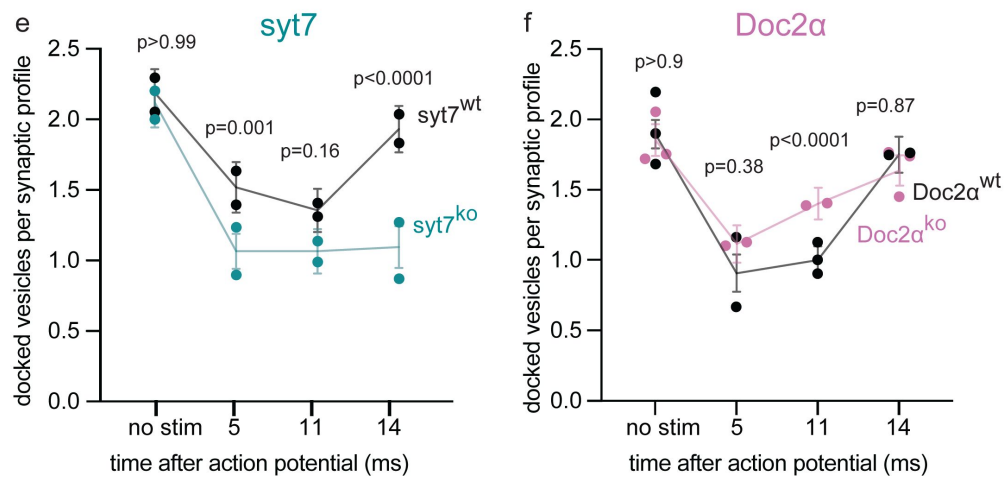


Fig. 6.

Syt7 is absolutely required for transient docking, while docked vesicles recover more quickly without Doc2α.

(A-D) Example transmission electron micrographs of syt7^{WT} (A), syt7^{KO} (B), Doc2α^{WT} (C), and Doc2α^{KO} (D) synapses frozen at the indicated time points after an action potential (AP) or without stimulation (no stim). Arrows indicate docked vesicles (synaptic vesicles with no visible distance between their membrane and the plasma membrane).

(E) The number of docked vesicles per synaptic profile (part of the synapse captured in a 2D section) in syt7 wild-type littermate controls and knockouts. Error bars indicate 95% confidence interval of the mean for all data pooled together; each dot indicates the mean from a single biological replicate. *P* values are from comparisons between wild-type controls (no stim, *n* = 217; 5 ms, *n* = 216; 11 ms, *n* = 217; 14 ms, *n* = 230 synaptic profiles) and knockouts (no stim, *n* = 193; 5 ms, *n* = 215; 11 ms, *n* = 230; 14 ms, *n* = 212 synaptic profiles) frozen at the same time point.

(F) same as E, but for Doc2α wild-type controls (no stim, *n* = 327; 5 ms, *n* = 229; 11 ms, *n* = 336; 14 ms, *n* = 192 synaptic profiles) and knockouts (no stim, *n* = 330; 5 ms, *n* = 231; 11 ms, *n* = 352; 14 ms, *n* = 321 synaptic profiles).

Number of biological replicates (from separate cultures frozen on different days and analyzed in separate randomized batches): 2 for each of the syt7 time points, 2 for 5 ms and 14 ms Doc2 WT, 2 for 5 ms and 11 ms Doc2 KO, 3 for Doc2 WT no stim and 11 ms, 3 for Doc2 KO no stim and 14 ms. The numbers of pits between wild type and knockout were compared using Brown-Forsythe ANOVAs with post-hoc Games-Howell's multiple comparisons tests. In pairwise tests, the same time point for wild type vs. knockout as well as each time point within each genotype were compared against each other (only comparisons between the same time point for wild type vs. knockout are shown, see Supplementary Table 2 for all pairwise comparisons). See [Fig. S6A](#) for active zone sizes from each condition, [Fig. S6C](#) for docked vesicle data normalized to the size of active zones, and [Fig. S6D](#) for distribution of undocked vesicles.

We then assessed synaptic vesicle exocytosis by quantifying the appearance of exocytic pits following a single action potential (1 ms, 1.2 mM external Ca²⁺, 37 °C) and freezing the neurons at 5, 11, and 14 ms after the action potential. These time points capture synchronous fusion, asynchronous fusion, and transient docking, respectively ([Kusick et al., 2020](#); [Li et al., 2021](#)). Consistent with our previous findings ([Kusick et al., 2020](#); [Li et al., 2021](#)), in WT controls exocytic pits peaked at 5 ms after an action potential, declined to less than half of this peak at 11 ms, then further declined to baseline by 14 ms ([Fig. 5E,F](#); pits in the active zone per synaptic profile: syt7 WT: no stim: 0.03 pits; 5 ms: 0.22 pits; 11 ms: 0.13 pits; 14 ms: 0.04 pits; Doc2α WT: no stim: 0.021 pits; 5 ms: 0.35 pits; 11 ms: 0.08 pits; 14 ms: 0.04 pits. See [Fig. S6A](#) for active zone sizes from each condition and [Fig. S6B](#) for pit data normalized to the size of active zones; see Supplementary Table 1 for full descriptive statistics and test statistics). In syt7 KOs, this sequence was unchanged: the number of pits at each time point in the KO was not different from the corresponding WT control (pits per synaptic profile: syt7 KO: no stim: 0.04 pits; 5 ms: 0.28 pits; 11 ms: 0.14 pits; 14 ms: 0.06 pits; *P* > 0.9 for each compared to the corresponding syt7 WT time point). By contrast, in Doc2α KO synapses, the number of pits at 5 ms was unchanged, but pits at 11 ms were almost completely abolished (pits per synaptic profile: Doc2α KO: no stim: 0.02 pits; 5 ms: 0.32 pits; 11 ms: 0.03 pits; 14 ms: 0.01 pits; *P* = 0.04 between WT and KO 11 ms, *P* = 0.02 between WT no stim and 11 ms, *P* > 0.9 between KO no stim and 11 ms). This phenotype mirrors the effect of EGTA-AM we previously reported ([Kusick et al., 2020](#)), consistent with Doc2α driving a large fraction of residual Ca²⁺ dependent AR. In sum, we obtained the same result from three different techniques (iGluSnFR imaging, patch-clamp electrophysiology, and zap-and-freeze electron microscopy): after a single action potential, Doc2α accounts for 54-67% of AR at hippocampal excitatory synapses, whereas deleting syt7 has no effect.

Is syt7 required for activity-dependent transient docking of synaptic vesicles? To test this possibility, we quantified all vesicles within 100 nm of the plasma membrane at the active zone. The distribution of undocked vesicles did not detectably change in any of the genotypes, nor at any of the time points after stimulation (**Fig. S6D**). WT controls showed rapid depletion and replenishment of docked vesicles after an action potential, as previously reported (Kusick et al., 2020; **Fig. 6E,F**). At 5 and 11 ms, there were ~40% fewer docked vesicles than in the no stimulation controls, then at 14 ms docked vesicles returned to baseline (**Fig. 6E,F**; docked vesicles per synaptic profile: syt7 WT: no stim: 2.19; 5 ms: 1.52; 11 ms: 1.36; 14 ms: 1.93; Doc2a WT: no stim: 1.90; 5 ms: 0.91; 11 ms: 1.00; 14 ms: 1.62. See **Fig. S6A** for active zone sizes from each condition and **Fig. S6C** for docked vesicle data normalized to the size of active zones; see Supplementary Table 2 for full descriptive statistics and test statistics). In syt7 KO neurons, consistent with our previous data (Vevea et al., 2021), there were ~25% fewer docked vesicles than in WT controls at 5 ms and 11 ms. At 14 ms, docked vesicles completely failed to recover in syt7 KO, remaining at less than half of the no-stim control (docked vesicles per synaptic profile: syt7 KO: no stim: 2.12; 5 ms: 1.07; 11 ms: 1.07; 14 ms: 1.09; $P < 0.0001$ between WT and KO at 14 ms, $P = 0.001$ at 5 ms between WT and KO, $P = 0.16$ at 11 ms between WT and KO). These data indicate that syt7 is absolutely required for transient docking of synaptic vesicles.

In Doc2a KO neurons, by contrast, we observed normal docked vesicle depletion and faster recovery (**Fig. 6F**). The number of docked vesicles was 40% greater in the KO than in the WT at 11 ms, about halfway towards full recovery to baseline (docked vesicles per synaptic profile: Doc2a KO: no stim: 1.85; 5 ms: 1.11; 11 ms: 1.40; 14 ms: 1.63; $P < 0.0001$ between WT and KO at 11 ms, $P < 0.0001$ between 11 ms and no stim in the KO). This is consistent with the idea that attenuation of AR spares some vesicles for ongoing synchronous release (Otsu et al., 2004; Yao et al., 2011), which could in part explain the slower train depression in Doc2a KOs (**Fig. 3** and **4**). We conclude that transient docking completely fails in the absence of syt7, while Doc2a is dispensable for docked vesicle recovery. Taken together with our data on exocytosis, these results suggest that syt7 supports synchronous and asynchronous release after multiple stimuli by triggering transient docking, while Doc2a is a Ca^{2+} sensor for asynchronous fusion itself.

Model for sequential action of syt7 and Doc2a during asynchronous release

To test whether a reaction scheme in which syt7 and Doc2a act sequentially is consistent with the physiological (**Fig. 1**) and morphological (**Fig. 5**) phenotypes observed in this work, we constructed a mathematical model. We aimed to investigate whether the theory proposed above could replicate the experimental data, though we cannot rule out that other (unexplored) model implementations match the experimental data similarly well. In the model, syt7 promotes Ca^{2+} -dependent synaptic vesicle docking and Doc2a is a high affinity Ca^{2+} -sensor for synaptic vesicle fusion (see **Fig. 7A** for simplified scheme, and **Fig. S7** for full reaction scheme). As in previously proposed models (Miki et al., 2016; Neher and Brose, 2018; Pan and Zucker, 2009; Walter et al., 2013; Weingarten et al., 2022), we assumed docking of synaptic vesicles to occur in two-steps: first synaptic vesicles are tethered to release sites before they dock and become fusion competent (**Figure 7A**). Vesicle exocytosis in the model depends on the total number of Ca^{2+} ions bound to syt1 and Doc2a per vesicle/release site, and is simulated using an adaptation of previously developed dual-sensor models (Kobbersmed et al., 2020; Sun et al., 2007). In this model each Ca^{2+} ion bound to either sensor was assumed to lower the energy barrier for vesicle fusion. This means that the rate of fusion increases exponentially as more Ca^{2+} ions are bound to syt1 and Doc2a (Schotten et al., 2015). We assumed that the fusion rate is accelerated more when Ca^{2+} binds syt1 than Doc2a. In our model, we implemented the action of syt1 on the vesicular fusion rate following the five-site binding model (Dulubova et al., 2005), which captures the physiological Ca^{2+} -dependence of fast neurotransmitter release without explicit modelling of molecular interactions (Kobbersmed et al., 2022). The additional role of Doc2a was implemented by two additional, independent Ca^{2+} -binding sites, following previously

developed dual-sensor models (Kobbersmed et al., 2020 [↗](#); Sun et al., 2007 [↗](#)). This was chosen because deleting the fast syt in the Calyx of Held uncovered a slow Ca^{2+} -dependent fusion reaction dependence with a cooperativity of two (Sun et al., 2007 [↗](#)). We assumed that both proteins induce synaptic vesicle exocytosis by acting through a common substrate (e.g., a limited number of SNARE complexes or plasma membrane binding sites; Groffen et al., 2010 [↗](#)), which was not explicitly included in the model. The competition between Doc2 α and syt1 was implemented by limiting the total number of Ca^{2+} ions that could be bound to syt1 and Doc2 α to five ions per release site/synaptic vesicle.

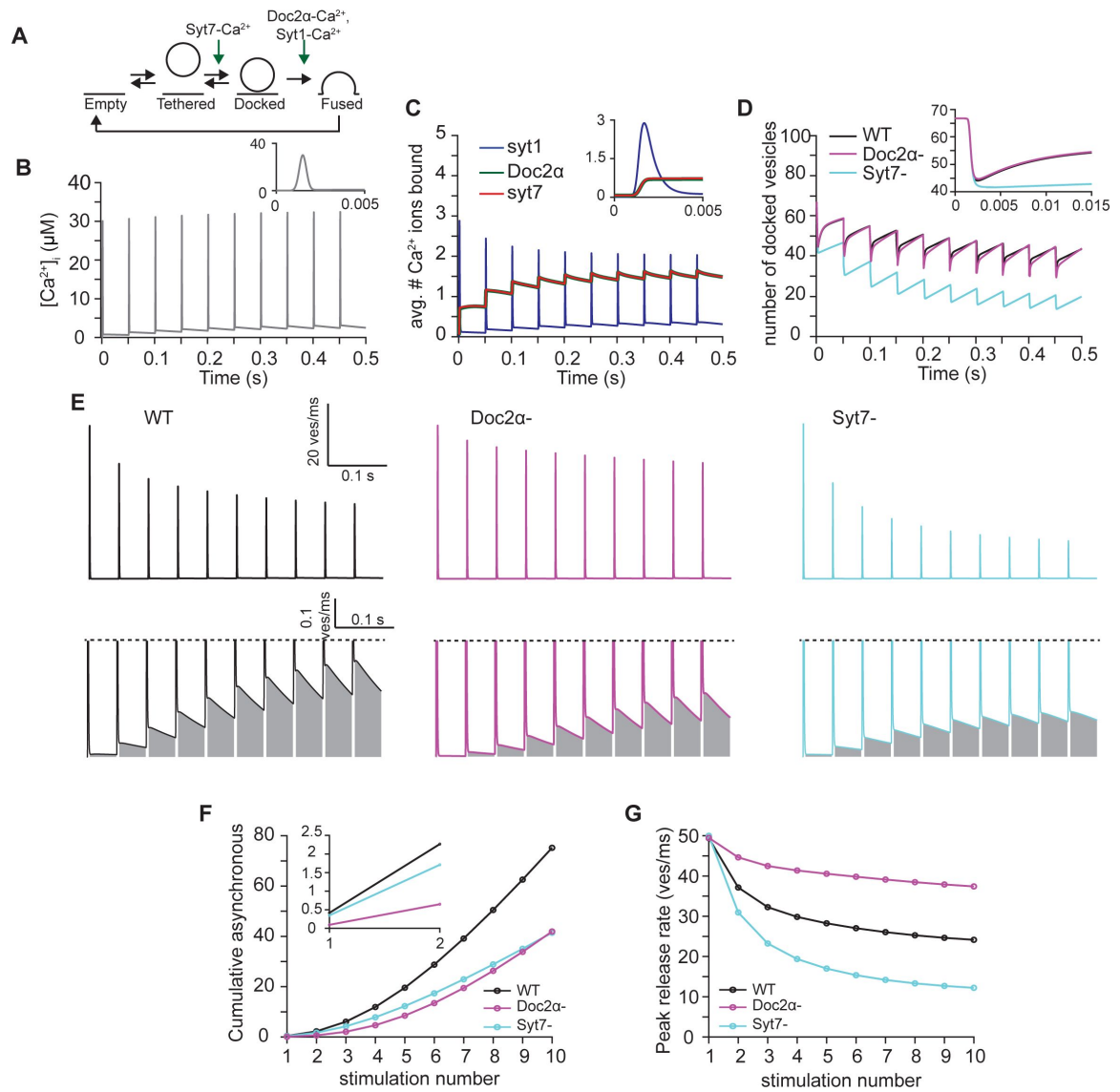


Fig. 7.

A mathematical model in which syt7 catalyzes synaptic vesicle docking and Doc2 α promotes fusion can qualitatively explain the observed phenotypes.

(A) Simplified scheme of the model in which syt7 acts as a catalyst of synaptic vesicle docking (increasing both docking and undocking rates) and Doc2 α regulates fusion together with syt1. All proteins only exert their effect when bound to Ca²⁺. In the model a release site can either be empty or occupied by a tethered or a docked vesicle. For full model scheme see [Fig. S7](#).

(B) Ca²⁺ transient used to perform the simulations. The transient corresponds to a signal induced by 10 APs at 20 Hz. Insert shows a zoom-in over the first 5 ms of the transient.

(C) Average number of Ca²⁺ ions bound to syt1, Doc2 α and syt7 over time. All syt1s expressed per synaptic vesicle can maximally bind five Ca²⁺-ions in total, Doc2 α and syt7 can each bind two Ca²⁺-ions per release site. Insert shows zoom-in over the first 5 ms of the simulation.

(D) The number of docked vesicles over time for simulations including functionality of all proteins (black, WT), simulations lacking Doc2 α (magenta, Doc2 α -) and simulations lacking syt7 (cyan, syt7-). Insert shows a zoom-in over the first 15ms of the simulation.

(E) Simulated release rates obtained using the full model (WT, left, black), the model lacking Doc2 α (Doc2 α -, middle, magenta) and the model lacking syt7 (syt7, right, cyan) simulations. Top line shows the entire trace. Bottom panels show a zoom-in to illustrate the asynchronous release component, which is indicated by the gray area.

(F) Cumulative number of asynchronously released vesicles per stimulus. Asynchronous release is quantified as the release between 5ms after the start of the AP and the start of the next AP. Insert shows zoom-in over the first two pulses.

(G) Peak release rates per stimulus for stimulations using the full model (WT, black), the model lacking Doc2 α (Doc2 α -, magenta) and the model lacking syt7 (syt7-, cyan).

Like Doc2 α and syt1, which reduce the energy barrier for exocytosis upon Ca²⁺ binding, we assumed that syt7 acts as a catalyst for docking ([Walter et al., 2013](#)). Because docking, unlike fusion, is reversible ([Kusick et al., 2020](#); [Murthy and Stevens, 1999](#); [Yavuz et al., 2018](#)), a reduction of the barrier height alone (without a change in the free energies of the docked or undocked states) increases both the docking and the undocking rates. Such a catalytic function of syt7 is consistent with the observations that 1) its loss did not affect steady state docking ([Fig 6](#)) and that 2) during transient docking in cultured hippocampal neurons, the number of docked vesicles returns to the pre-stimulation baseline. However, it is possible that Ca²⁺-bound syt7 instead selectively increases the docking rate or decreases the undocking rate (and such a mechanism may explain its role in facilitation: [Kobbersmed et al., 2020](#); [Lin et al., 2022](#); [Pan and Zucker, 2009](#); [Weingarten et al., 2022](#)).

Since both Doc2 α and syt7 are present on the plasma membrane ([Sugita et al., 2001](#); [Verhage et al., 1997](#); [Vevea et al., 2021](#); [Weber et al., 2014](#)), they were assumed to bind Ca²⁺ irrespective of whether the site is occupied by a vesicle (see methods). In this way, the release site (syt7 and Doc2 α) maintains its Ca²⁺-bound state independent of whether synaptic vesicles tether, dock, or fuse. This is highly relevant for Ca²⁺ sensors with high affinity but slow binding kinetics, like syt7 and Doc2 α ([Hui et al., 2005](#); [Xue et al., 2015](#); [Yao et al., 2011](#)) and distinguishes them from the vesicular sensor syt1, which is unlikely to bind Ca²⁺ on undocked synaptic vesicles ([Bai et al., 2004](#); [Radhakrishnan et al., 2009](#)).

In our model, we explicitly calculate Ca^{2+} association with and dissociation from syt1, syt7, and Doc2a. This causes variable reaction rates for synaptic vesicle docking, undocking and fusion, resulting in a large increase in the number of states a release site can be in and in the number of model parameters. Nevertheless, we decided on explicit simulation of Ca^{2+} binding to these proteins as this allowed us to integrate information on their Ca^{2+} -binding properties. Moreover, the explicit simulation of Ca^{2+} binding and unbinding is highly relevant in systems with rapid dynamics, as it takes time before the Ca^{2+} sensors reach equilibrium. This especially holds for the slow Ca^{2+} sensors syt7 and Doc2a. The affinity of syt1 and syt7 as well as the Ca^{2+} -binding rate of syt1 and its effect on the energy barrier were estimated from the literature (see Supplementary Table 3). For simplicity, Doc2a and syt7 were assumed to share the same Ca^{2+} -binding dynamics and that the docking/undocking rate is modulated by the association of Ca^{2+} to two syt7 binding sites at each release site. These simplifications reduced the number of unknown parameters in the model and ensured that syt7 and Doc2a have the same properties except for which energy barrier they exert their effects (docking vs. fusion). This allowed us to more specifically investigate whether differences in the reactions on which syt7 and Doc2a act can explain differences in the apparent roles these two proteins have in AR. Additionally, we assumed that the rates of undocking and untethering are equal to the rates of docking and tethering, respectively. This assumption was made to simplify parameter evaluation of the model. The remaining reaction rates and the effect syt7 and Doc2a exert on the energy barriers were manually adjusted to obtain good qualitative correspondence to the experimental data (see Methods, Supplementary Table 3 and [Fig. S8](#)). Please note that the selected parameter values do not provide a unique solution (as indicated by parameter space evaluation, [Fig. S9](#)), meaning that our modelling effort does not provide reliable estimates of the parameters used in the model.

With these parameters and assumptions, we evaluated the model during a stimulus train. We used estimated Ca^{2+} transients resulting from 20 Hz AP trains to drive our model ([Fig. 7B](#)) and monitored Ca^{2+} -binding to Doc2a, syt7 and syt1, the number of docked vesicles, the peak rate of synchronous release, and the magnitude of AR. Our simulations indicate that all three sensors reach maximum Ca^{2+} -activation at a similar time ([Fig. 7C](#)), despite slower Ca^{2+} -binding rates of syt7 and Doc2a compared to syt1. However, syt1's low Ca^{2+} affinity but fast binding kinetics ensure that its Ca^{2+} association closely follows the shape of the Ca^{2+} -transient itself ([Fig. 7C](#)). The activation of syt1 by Ca^{2+} dominated the synchronous release observed immediately after the first AP, as removal of syt7 and Doc2a from the model did not affect the peak release rate ([Fig. 7G](#)). This only holds if Doc2a has a ~40% lower contribution to reducing the fusion barrier upon Ca^{2+} binding compared to syt1 ([Fig. 7G](#), [Fig. S8](#)). By contrast, AR in response to a single AP strongly depended on the delayed and sustained activation of Doc2a ([Fig. 7C](#) and [F](#)). Although Doc2a and syt7 have the same Ca^{2+} binding dynamics in the model, syt7 did not contribute noticeably to neurotransmitter release induced by the first AP ([Fig. 7F](#) and [G](#)) due to its assigned role in docking. These results from the first action potential during train stimulation are consistent with the experiments ([Fig. 1](#) and [Fig. 2](#)).

During the stimulus train in simulations of the control condition, synchronous release gradually decreased, while AR showed the opposite trend ([Fig. 7F](#) and [G](#)). This behavior is influenced by the competition between Ca^{2+} -bound syt1 and Doc2a for a common recourse to elicit their effect on the fusion barrier ([Fig. 7C](#)). Removal of Doc2a from the model caused a reduced depression of synchronous release and reduced AR (Compare [Fig. 7E](#), [G](#) with [Fig. 3D](#) and [4B](#)). These results are consistent with the experiments ([Fig. 3](#) and [4](#)) and suggest that, though less efficient in promoting fusion, Doc2a-mediated fusion has increasingly more influence on synaptic transmission during extended AP trains.

As in the experiments, the physiological relevance of syt7's ability to catalyze vesicle docking was revealed only from the second stimulus onward. Owing to its higher Ca^{2+} affinity and release site localization, syt7, like Doc2a, became increasingly activated over the duration of the stimulus train ([Fig. 7C](#)). Ca^{2+} -bound syt7 ensured a fast recovery of docked vesicles after depletion

(independent of whether fusion depended on Doc2 α and Syt1 or Syt1 alone, **Fig. 7D**). As observed experimentally in the syt7 KO (**Fig. 6**), removal of this catalytic function in the model resulted in very limited recovery of docked vesicles before the next stimulus (**Fig. 7D**). Because under prolonged stimulation both synchronous and asynchronous release depend on vesicle replenishment, removal of syt7 from the model resulted in reduction of both release modes (**Fig. 7E, G**). This is in agreement with the syt7 KO data (**Fig. 3** and **4**). Thus, there is a good qualitative correspondence of our models to the experimental data. This demonstrates that the results of our experiments are consistent with a sequential role of syt7 and Doc2 α in promoting synaptic vesicle docking and fusion, respectively.

Epistasis experiments indicate that syt7 and Doc2 α act sequentially

The sequential model predicts that syt7 works upstream of Doc2 α to support AR. If syt7 and Doc2 α act as parallel Ca²⁺ sensors triggering AR directly, rather than in sequence through transient docking (syt7) and fusion (Doc2 α), deleting both proteins would be expected to reduce or eliminate the remaining AR from either of the single knockouts. To test this prediction, we performed an epistasis experiment using double knockout (DKO) mice. For these experiments we conducted hippocampal slice electrophysiology. We first applied single action potentials and observed that loss of both proteins was not additive with respect to AR: the reduction in slow EPSC charge was identical in Doc2 α neurons compared to Doc2 α /syt7 DKO (**Fig. 8A,B**). Again, EGTA-AM served as a control. This was the expected result, as syt7 single KO had no effect on responses to single action potentials. We then applied train stimulation (**Fig. 8C**) and again quantified the tonic charge transfer component as a measure of AR (**Fig. 8D**). Importantly, no differences in AR were observed between the Doc2 α KO and the Doc2 α /syt7 DKO condition. However, the DKO showed a different short-term plasticity phenotype than either single KO. Synchronous release early in the train was similar in DKO to EGTA-treated neurons, with abolished paired-pulse facilitation but not the enhanced depression seen in syt7 KOs (**Fig. S11**). This is in line with the model and other experiments showing competition between synchronous and asynchronous release, such that deleting Doc2 α slows depression. Note that in EGTA-treated neurons, like in the DKO, both residual Ca²⁺-triggered docking and AR are reduced (Kusick et al., 2020), explaining why the DKO short-term plasticity phenotype is so similar to the effects of EGTA. However, in the DKO, late in the train amplitudes decayed to the same lower level as syt7 KOs, suggesting a specific role for syt7 in limiting steady-state depression (**Fig. S7**). Together, these findings strongly support a sequential model for the action of syt7 and Doc2 α (illustrated in **Fig. S12**, as detailed below).

Consistent with the experimental data, removal of both syt7 and Doc2 α from the model resulted in less depression of synchronous release compared to the simulations lacking syt7 only (**Fig. S10**). This is because 1) Ca²⁺-bound syt1 gains more access to the fusion machinery in this phase when not competing with Doc2 α and 2) the reduced asynchronous release (in both Doc2 α KO and DKO) causes more vesicles to be available for synchronous transmission when they are “spared” from AR. This matches the experimental observations (**Fig. S11**). At the same time, the model shows reduced asynchronous release after 10 action potentials in simulations lacking both syt7 and Doc2 α , compared to simulations in which only one of these proteins had been removed (**Fig. S10C**). This is in contrast to the experimental data, which detected no difference in the cumulative charge of DKO compared to Doc2 α KOs after 50 action potentials (**Fig. 8D**). This could be due to some simplifications that were made while constructing the model such as 1) the implicit simulation of competition between Doc2 α and syt1, 2) neglecting potential Ca²⁺-independent roles of these proteins (see **Discussion**), or 3) the fact that Doc2 α is the only slow Ca²⁺ sensor promoting fusion in the model, while the remaining AR in the DKO experiments points to another unidentified sensor. Nonetheless, our modelling confirms that the sequential action of syt7 and Doc2 α in the Ca²⁺-dependent docking and fusion of synaptic vesicles, respectively, can explain the phenotypes observed in Doc2 α and syt7 KO neurons, and partially explain the phenotype of the DKO neurons.

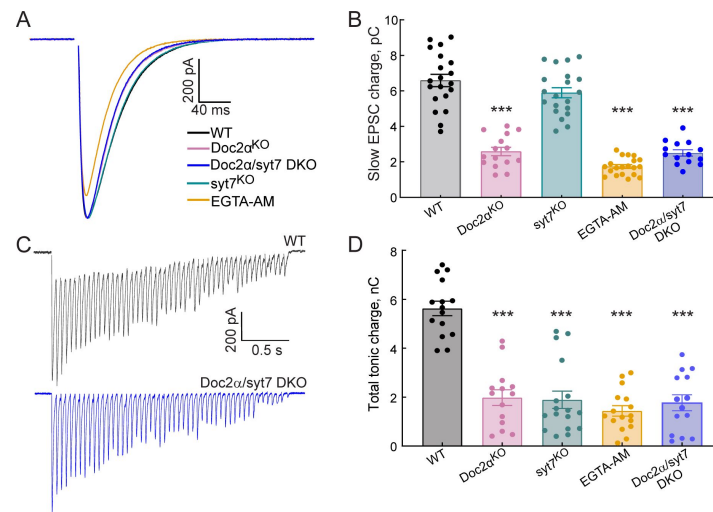


Fig. 8.

AR in Doc2α/syt7 DKO neurons: non-additive effects on AR indicate function through a common pathway.

All recordings were carried out using acute hippocampal slices, in CA1, as described in [Fig. 2](#) and [4](#).

(A) Averaged traces of evoked EPSCs recorded from WT (n=18 recordings; from 5 independent litters), Doc2αKO (n=15; from 4 independent litters), Doc2α/syt7 DKO (n=14; from 4 independent litters), and EGTA-AM treated neurons (n=20; from 4 independent litters).

(B) Bar graph summarizing the slow EPSC charge values. One-way ANOVA, followed by Dunnett's test, was used to compare mutants against WT. **, and *** indicate p < 0.01, and p < 0.001.

(C) Representative EPSCs triggered by 20 Hz stimulus trains using WT and Doc2α/syt7 DKO (n=15 recordings; from 5 independent litters) neurons.

(D) AR in WT, Doc2αKO, syt7KO, Doc2α/syt7 DKO, and EGTA-AM treated neurons was estimated by measuring the total tonic charge transfer during the 20 Hz stimulus train, as described in [Fig. 4C](#).

Discussion

Resolving the asynchronous release Ca^{2+} sensor controversy

Both Doc2 α (Yao et al., 2011 [DOI](#)) and syt7 (Wen et al., 2010 [DOI](#)) have been implicated in AR, but conflicting results over the years led to an unresolved debate - which sensor mediates AR? Several factors may have contributed to these inconsistent results, including stimulation frequency, the type of synapse under study, and method used to measure release. To minimize the effects from these different factors, we conducted a side-by-side comparison of Doc2 α and syt7 KO neurons, using direct optical measurements of glutamate release, patch clamp electrophysiology, and ultrastructural characterization of millisecond-by-millisecond vesicle dynamics from mouse hippocampal neurons. These experiments show that Doc2 α drives significant levels of AR after a single action potential. In sharp contrast, knocking out syt7 has a negligible effect on this mode of release in response to a single action potential. Remarkably, during a stimulus train, both Doc2 α and syt7 are equally important for AR.

These results resolve some of the inconsistencies in the literature. First, Doc2 α is a Ca^{2+} sensor for AR both after a single stimulus, and also during stimulus trains in both cultured hippocampal neurons and Schaffer collaterals. Second, syt7 contributes to AR, but its role is only apparent from the second action potential onward. Thus, at least in the excitatory mouse hippocampal synapses studied here, these sensors both contribute to AR. However, it is still likely that which sensor(s) are required depends on synapse type, since Doc2 α played no apparent role in transmission at Purkinje cell to deep cerebellar nuclei synapses (Khan and Regehr, 2020 [DOI](#)). This, along with our finding that syt7/Doc2 α DKO still had remaining AR, indicates that there are other Ca^{2+} sensors for AR. Synaptotagmin 3, a faster high-affinity sensor, was recently proposed to support facilitation and resistance to depression by recruiting docked synaptic vesicles, in much the same manner we described here for syt7 (Weingarten et al., 2022 [DOI](#)). It stands to reason that syt3 could also similarly support AR during repetitive activity. There are likely also sensors yet to be identified that, like Doc2, directly drive AR. Thus, further work should explore the diversity of synaptic Ca^{2+} sensors and how they contribute to heterogeneity in synaptic transmission throughout the brain.

Physiological relevance of rapid activity-dependent docking triggered by syt7

How syt7 contributes to three separate phenomena, paired-pulse facilitation, AR, and resistance to synaptic depression, is a major question in the field (Husson and Regehr, 2020 [DOI](#); Jackman and Regehr, 2017 [DOI](#); Liu et al., 2014 [DOI](#); Vevea et al., 2021 [DOI](#)). Our data suggest that syt7 drives activity-dependent transient docking to support all three processes. In the case of paired-pulse facilitation it can supply docked vesicles for syt1-mediated synchronous release; it likely functions in the same manner to reduce synaptic depression during train stimulation. In the case of AR, syt7-mediated docked vesicles can be used by Doc2 α , which then directly triggers this slow mode of transmission. This is consistent with the recent proposal that activity-dependent docking underlies both short-term plasticity and AR (Kobbersmed et al., 2020 [DOI](#); Miki et al., 2018 [DOI](#), 2016 [DOI](#); Neher and Brose, 2018 [DOI](#); Silva et al., 2021 [DOI](#)), though see (Mendonça et al., 2022 [DOI](#)) for a correlation between short-term plasticity and AR with a different proposed mechanism.

Three pieces of data reported here support this notion. First and foremost, transient docking, which appears to mirror the time course of paired-pulse facilitation (Kusick et al., 2020 [DOI](#)), is completely blocked in syt7 KO hippocampal neurons. Consequently, these synapses do not facilitate, instead depressing quickly and profoundly, suggesting that syt7-mediated docking is at least in part necessary for facilitation. Second, despite release probability not changing, docked vesicles are more severely depleted (~33% compared to wild type at 5 ms after an action potential) in syt7 KO both after single action potentials (this study) and high-frequency trains

(Vevea et al., 2021 [DOI](#)). This implies that vesicles are normally recruited by syt7 to the active zone continuously during the first 15 ms after stimulation in WT synapses. Third, in the absence of the asynchronous Ca^{2+} sensor Doc2a, the recovery of docked vesicles is significantly faster, nearly returning to baseline by 11 ms, suggesting that some vesicles docked by syt7 are fused by Doc2a. Although we cannot rule out the possibility that Doc2a works by docking and then fusing vesicles that are initially undocked, slowed train depression of synchronous release and faster docked vesicle replenishment in Doc2a KO suggests vesicles are still being recruited in the absence of Doc2a, but are less likely to fuse asynchronously. Thus, these data show that syt7-mediated docking contributes to both AR and short-term plasticity. We tested our model for the sequential action of syt7 and Doc2a by conducting an epistasis experiment using DKO mice. Disruption of syt7 did not exacerbate the loss of AR in Doc2a KOs either after single stimuli or train stimulation. These findings are consistent with the idea that syt7 functions upstream of Doc2, with the upstream step (transient docking) required only during sustained activity (summarized in the model shown in **Fig. S8** [DOI](#)).

There are important questions posed by this sequential idea. First, while we found no statistically significant effect of syt7 KO on responses to a single action potential in both the hippocampal preparations used in this study, we previously found a very small (2%) but statistically significant decrease in AR in the KO (Vevea et al., 2021 [DOI](#)). By contrast, syt7 is required for the majority of AR after single action potentials at cerebellar granule cell synapses (Turecek and Regehr, 2018 [DOI](#)). Why does syt7 sometimes contribute substantially to responses to single stimuli: is this because transient docking is more important for initial responses at some synapses than others, or is syt7 actually able to trigger synaptic vesicle fusion? Second, the biochemical basis for the different functions of Doc2 and syt7 is unknown: what distinguishes the ability of a Ca^{2+} sensor to trigger docking from its ability to trigger fusion? Curiously, syt7, unlike the fast syts and like the other apparent ‘docking sensor’ syt3 (Weingarten et al., 2022 [DOI](#)), is strongly enriched not on synaptic vesicles but at the plasma membrane (Liu et al., 2014 [DOI](#); Veva et al., 2021 [DOI](#)). In fact, it is non-functional when directed to synaptic vesicles (Vevea et al., 2021 [DOI](#)). This is in contrast to syt7’s actions outside the synapse and in non-neuronal cells, where it localizes to dense-core vesicles and lysosomes and seems to trigger exocytosis directly (Chakrabarti et al., 2003 [DOI](#); Tawfik et al., 2021 [DOI](#); van Westen et al., 2021 [DOI](#)). These are all hints that targeting to the plasma membrane may be relevant to a role in Ca^{2+} -triggered docking as opposed to fusion. Still, the mechanism by which syt7 helps promote vesicle docking in response to Ca^{2+} remains to be discovered.

A sequential model of syt7 and Doc2 in transmitter release

We confirmed that the dynamics observed in the experimental data could be qualitatively recapitulated by a mathematical model in which syt7 and Doc2a act to promote synaptic vesicle docking and fusion, respectively. Both experiments and modeling showed: a) that syt7-mediated docking promotes synchronous and AR from the second stimulus onwards, and b) that Doc2a promotes AR at the cost of synchronous release. In this work we presented one way to implement the theory. We cannot exclude the possibility that other implementations can also explain the dynamics of the number of docked vesicles and synaptic fusion events observed in the experimental data.

In our model, syt7 acts as a catalyst for synaptic vesicle docking, meaning that Ca^{2+} -bound syt7 enhances both docking and undocking rates. This ensures that the number of docked vesicles at rest is independent of the presence of syt7, accounting for the observation that steady-state docking is normal in syt7 KO neurons (Vevea et al., (2021) [DOI](#); this study). If syt7 functions as a docking catalyst, the number of docked vesicles cannot overshoot during repetitive stimulation, but only return to baseline. While this is consistent with the EM data presented here, it does not explain the established role of syt7 in facilitation (Jackman et al., 2016 [DOI](#)), as facilitation has been proposed to depend on an *increase* in the number of docked/primed vesicles (Jusyte et al., 2023 [DOI](#); Kobbersmed et al., 2020 [DOI](#); Miki et al., 2016 [DOI](#); Neher and Brose, 2018 [DOI](#)). Therefore, it remains possible that syt7 selectively alters docking or undocking rates. Corresponding to the role of syt7 in

our model, another model previously showed that syt3 promotes Ca^{2+} -dependent docking (Weingarten et al., 2022). As in our model, as well as various other models (Miki et al., 2016; Pan and Zucker, 2009; Pulido and Marty, 2018; Walter et al., 2013), refilling of the readily releasable pool of vesicles occurred in two steps: vesicles first enter a state from which no fusion can occur (the tethered state in the model presented here). Subsequently, these vesicles become fusion competent (the docking reaction in the model presented here). It is also possible that some of syt7's role in making vesicles fusion-competent is not observable by docking in our electron microscopy experiments. At the moment it is difficult to know, since the cultured hippocampal neurons we used only facilitate very slightly (Figure 3). Future studies should prioritize zap-and-freeze experiments in intact circuits on synapses with high paired-pulse ratios to test if an overshoot in docking is actually responsible for synaptic facilitation.

Synaptic vesicle exocytosis in the model was simulated following the dual-sensor model (Kobbersmed et al., 2020; Sun et al., 2007), in which Ca^{2+} binds to two different sensors promoting fusion (Doc2a and syt1 in the model presented here). We diverged from the dual-sensor model by implementing a competition between syt1 and Doc2a for Ca^{2+} binding. This competition was needed to recapitulate the reduced depression observed in Doc2aKO neurons compared to WT controls (Fig. 3 and 4). The direct competition between Doc2a and syt1 could indicate a need to act at a limited number of “slots” at the release site (e.g., SNARE complexes or lipid clusters; Chen et al., 2021; Groffen et al., 2010; Honigsmann et al., 2013; Kobbersmed et al., 2022; Yao et al., 2011; Zhou et al., 2015). Additionally, the apparent competition between AR and synchronous release over a limited supply of docked vesicles (as proposed by Otsu et al., 2004 and Yao et al., 2011, and suggested by the EM here) may play a role in the slower depression of synchronous release observed in Doc2a KOs. Which of these two forms of competition between synchronous and asynchronous release, over vesicles or over slots/molecules, is more important at a given synapse likely depends on: a) how limited the supply of docked vesicles is and b) the relative copy numbers of Doc2a/syt1 in release sites. Our model describes the association to ‘slots’ implicitly by lumping it together with Ca^{2+} binding and synaptic vesicle fusion. This also means that our model can only account for Ca^{2+} -dependent functions of the included proteins. Recent analysis, however, indicates that syt1 pre-associates with these slots at resting Ca^{2+} -concentrations, which would limit access to these slots for other sensors (Kobbersmed et al., 2022). This arrangement would account for the reported clamping syt1 exerts on spontaneous transmission (Courtney et al., 2019; Kochubey and Schneggenburger, 2011; Schupp et al., 2016) and AR (Maximov and Südhof, 2005; Nishiki, 2004). Accordingly, there will be situations where the model falls short in providing an explanation. Nonetheless, it shows that sequential action of syt7 and Doc2a can explain the role these proteins have in docking, short-term plasticity, and asynchronous release in mouse hippocampal synapses.

Methods

All animal care was performed according to the National Institutes of Health guidelines for animal research with approval from the Animal Care and Use Committees at the Johns Hopkins University School of Medicine and the University of Wisconsin, Madison (Assurance Number: A3368-01). Both male and female syt7^{KO}, Doc2a^{KO}, and WT littermates were used.

Mouse lines used for this study were as follows:

- C57BL/6-Syt7^{tm1Nan} (28)
- C57BL/6-DOC2a^{em1Smoc} (27)
- C57BL/6-DOC2a^{em1Smoc};C57BL/6-Syt7^{tm1Nan}

Double knockouts were maintained as homozygous null. Single knockout lines were maintained as heterozygotes, except for double knockout electron microscopy experiments, in which *DOC2a*^{+/+} mice were used as the wild-type control.

Neuronal cell culture

For single-knockout experiments, *Doc2a* and *syt7* KO mice were maintained as heterozygotes, so that littermate controls could be used. These mice were bred, and postnatal day 0-1 pups were genotyped, with homozygotes for the null allele (-/-) and the wild-type allele (+/+) being used for culture. For double knockout experiments, mice were maintained as homozygotes, with *Doc2a*^{+/+} used as the wild-type control. For iGluSnFR experiments, WT and KO hippocampal neurons were grown in mass culture for 14 DIV. In brief, hippocampi were dissected from mouse brains and digested for 25 min at 37 °C in 0.25% trypsin-EDTA (Corning). After mechanical dissociation, hippocampal neurons were plated on 12 mm glass coverslips pretreated with poly-D-lysine (ThermoFisher). Neurons were cultured in Neurobasal A (GIBCO) based media with B27 (2%, ThermoFisher) and GlutaMAX (2 mM, GIBCO) at 37 °C with 5% CO₂.

For high-pressure freezing and electron microscopy, cell cultures were prepared on 6-mm sapphire disks atop a feeder layer of astrocytes, as previously described (Kusick et al., 2020 [DOI](#)). Astrocytes were harvested from cortices with the treatment of trypsin (0.05%) for 20 min at 37 °C, followed by trituration and seeding on T-75 flasks containing DMEM supplemented with 10% FBS and 0.2% penicillin-streptomycin. After 2 weeks, astrocytes were plated on 6-mm sapphire disks (Technotrade Inc) coated with poly-D-lysine (1 mg/ml), collagen (0.6 mg/ml) and 17 mM acetic acid at a density of 13 x 10³ cells/cm². Genotyping was performed either 1) after hippocampal dissection, using cortices lysed with 10 mM Tris-HCL pH8.0 and 100 mM NaCl in the presence of 50 µg proteinase K, with hippocampi either left in Neurobasal A at 37 °C before switching to papain after or left in dissection medium (1% penicillin-streptomycin, 1 mM pyruvate, 10 mM HEPES, 3 mM glucose in HBSS) at 4 °C, or 2) using tail clips (alkaline lysis; Truett et al., 2000 [DOI](#)) of live mice, which were then sacrificed and dissected after genotyping was finished. We note that leaving the neurons in dissection medium at 4 °C results in more consistent cell health than Neurobasal A at 37 °C, particularly when hippocampi are left for several hours while genotyping is performed. Hippocampi were dissected under a microscope and digested with 1) papain (0.5 mg/ml) and DNase (0.01%) in the dissection medium for 25 min at 37 °C with gentle perturbation every 5 min or 2) papain (0.5 mg/mL) in Neurobasal A with 200 rpm shaking. After trituration, neurons were seeded onto astrocyte feeder layers at a density of 20 x 10³ cells/cm² (75,000 per well) and cultured in Neurobasal A with 2% B27 and 2 mM at 37 °C with 5% CO₂. Before use, sapphire disks were carbon-coated with a “4” to indicate the side that cells are cultured on. The health of the cells, as indicated by de-adhered processes, floating dead cells, and excessive clumping of cell bodies, was assessed immediately before experiments.

Plasmids

-pF(UG) CamKII sf iGluSnFR A184V (29); Addgene #158769

-pF(UG) CamKII sf iGluSnFR S72A (26)

Lentivirus production and use

For viral transduction, the original (24, 29) and low-affinity glutamate S72A were used (25, 26). iGluSnFRs were subcloned into a FUGW transfer plasmid (Vevea and Chapman, 2020 [DOI](#)). Lentiviral particles were generated as previously described (Vevea et al., 2021 [DOI](#); Vevea and Chapman, 2020 [DOI](#)) by co-transfecting the transfer, packaging, and helper (pCD/NL-BH*ΔΔΔ and VSV-G encoding pLTR-G) plasmids into HEK 293/T cells. Lentivirus was collected from the media 48–72 h after transfection and concentrated by ultracentrifugation. Viruses were titrated and then used to infect neurons on DIV 3-5.

iGluSnFR imaging and quantification

iGluSnFR fluorescence intensity changes were recorded and analyzed as previously described (Vevea et al., 2021 [DOI](#); Vevea and Chapman, 2020 [DOI](#)), with slight adjustments to imaging time and binning. To measure iGluSnFR signals in response to a single stimulus, the medium-affinity iGluSnFR (A184V) was used in **Fig. 1A-D** [DOI](#) and **S1** [DOI](#). For train stimulation, the low-affinity iGluSnFR sensor (S72A) was used because it more effectively resolved individual responses from high-frequency stimulation due to its faster decay time (Vevea et al., 2021 [DOI](#)). Imaging was performed in media (305 mOsm) comprising (in mM): 128 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 30 D-glucose, and 25 HEPES, pH 7.3, with D-AP5 (50 μ M) (Abcam; ab120003), CNQX (20 μ M) (Abcam; ab120044), Picrotoxin (100 μ M) (Tocris; 1128) added to prevent recurrent excitation. Single image planes were acquired (2x2 binning) with 10 ms exposure (100 Hz) using 488 nm excitation and 520 emission. Single planes were selected so that the highest amount of iGluSnFR expressing dendrite arbor was in focus. Laser intensity was set so that the amplitude of iGluSnFR peaks ($\Delta F/F_0$) from a 0.5 Hz field stimulus did not change over the course of at least 20 s (photobleaching error). For single stimuli imaging, 200 frames (10 ms exposure/2 s total) were collected and a single field stimulus was triggered at 500 ms after the initial frame. For train stimuli imaging, 50 stimuli at 10 Hz (5 s) or 20 Hz (2.5 sec) were applied via field stimulation, and 500 or 750 frames, each with a 10 ms exposure time, were collected. Single and train (10 Hz) stimuli (1 ms square pulses) were triggered by a Grass SD9 stimulator through platinum parallel wires attached to a field stimulation chamber (Warner Instruments; RC-49MFSH). Voltage for field stimulus was set to the lowest voltage that reliably produced presynaptic Ca²⁺ transients in most (>95%) presynaptic boutons (5–30 V). All iGluSnFR imaging experiments were performed at 33–34°C. Temperature and humidity were controlled during experiments by a Tokai incubation controller and chamber. Glutamate release signals were defined as fluorescence intensity changes that were > five times the standard deviation of the noise (**Fig. S1C** [DOI](#)). Timing of release was determined based on the frame in which the signal peaked, including for dual peaks in the case of synchronous and asynchronous release at the same bouton. For all iGluSnFR experiments, we initially recorded 500 ms as the baseline; the mean intensity was defined as F₀. iGluSnFR fluorescence amplitude changes are reported as $\Delta F/F_0$. Throughout this study, we term regions of interest as boutons because these regions were defined as yielding at least one iGluSnFR response during the first three stimuli of a 10 Hz train. iGluSnFR data were analyzed using a custom ImageJ plugin (Vevea and Chapman, 2020 [DOI](#)) as follows: the macro begins by subtracting the average projection intensity (the pre-stimulation baseline) from a maximum intensity projection (the largest intensity from the entire image series). The result was duplicated, and the mean was filtered by an area of a radius of 10 pixels. The copied image is converted to a 32-bit image for subsequent calculations. The original duplicated and filtered images are subtracted, creating a new image (“Result”). Thresholding is applied to the “Result” image, converting it to a binary image. Watershed segmentation was then used to separate touching objects. The results were copied and imported into Axograph X 1.7.2 (Axograph Scientific), where traces were normalized, and background subtracted using pre-stimulus data. All iGluSnFR imaging and analysis was performed blind to genotype.

Brain slice preparation

Acute hippocampal slices were prepared from mice aged between P30 and P60. Animals were anesthetized using isoflurane and then rapidly decapitated. Brains were quickly removed and placed into ice-cold NMDG-HEPES artificial cerebral spinal fluid (aCSF; 300–310 mOsm) comprising (in mM): 92 N-Methyl-D-glucamine (NMDG), 2.5 KCl, 1.25 NaH₂PO₄, 30 NaHCO₃, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 CaCl₂, and 10 MgSO₄, and 20 HEPES, pH 7.3. This solution was continuously bubbled with 95% O₂ and 5% CO₂. Three hundred μ m thick coronal brain sections containing the hippocampal region were cut using a vibratome (Leica VT1200, Leica, Germany). To avoid any potential recurrent excitation, a sharp cut was made between CA3 and CA1. Slices were placed in a chamber and allowed to recover in 150 ml artificial cerebral spinal fluid at 34 °C for 25 min. Sodium (2 M stock in artificial cerebral spinal fluid) was added back at the following time intervals: 0 min (250 μ l), 5 min (250 μ l), 10 min (500 μ l), 15 min (1 ml),

and 20 min (2 ml) during the 25 min recovery incubation. Slices were then gently placed into another chamber containing aCSF, but with 92 mM NMDG replaced with 92 mM NaCl, at room temperature (at least 1 hour) before being transferred to the recording chamber.

Electrophysiological recordings in acute hippocampal slices

Whole-cell voltage-clamp recordings were performed under an upright microscope (BX51WI, Olympus, Melville, NY) equipped with a water-immersion objective (LUMPlanFL N 40x/0.8 W; Olympus, Melville, NY). Hippocampal slices were continuously perfused (2 ml/min) with carbogen-saturated, modified aCSF (in mM: 125 NaCl, 25 NaHCO₃, 25 glucose, 2.5 KCl, 2 CaCl₂, 1.25 NaH₂PO₄, and 1 MgCl₂, adjusted to 300-310mOsm). For perfusion, aCSF was heated to 34°C using a Single Channel Temperature Controller (TC-324C, Warner Instruments, Holliston, MA). Recording pipettes were prepared from borosilicate glass (1.5 mm diameter glass capillary, Sutter Instrument, Novato, CA) using a two-step puller (PP-830, Narishige, Japan), and were polished using an MF-830 micro forge (Narishige, Japan) immediately prior to use. Pipette position was controlled using an MP-285 motorized micromanipulator (Sutter Instrument, Novato, CA); the resistances were typically 4-6 MΩ when filled with recording solution consisting of (in mM): 135 K-gluconate 5 KCl, 10 HEPES, 1 EGTA, 6 Na₂ phosphocreatine, 0.3 Na₃GTP and 2 MgATP. To chelate residual Ca²⁺, EGTA-AM (Millipore, Burlington, Massachusetts) was added to the bath at a final concentration of 25 μM for 30 minutes; neurons were then allowed to recover for another 20 minutes before being placed in the recording chamber. Neurons in the CA1 region were visualized using a Retiga electro CCD camera (ELEDYNE PHOTOMETRICS, Tucson, AZ). Patched neurons were held at -70 mV, with access resistance (Ra) less than 20 MΩ throughout the recordings. A concentric bipolar electrode (FHC, 125/50 μm extended tip) was placed in the stratum radiatum (Schaffer collateral fiber) ~300-500 μm away from the recording pipette (note that this large bore diameter is responsible for the large currents in our recordings). Stimulation strength was selected by starting at 0.5-1.5 mA, steadily increasing stimulation strength and adjusting electrode position until stable currents from 1 Hz test stimuli were achieved. EPSCs were pharmacologically isolated by bath application of 50 μM D-APV and 20 μM bicuculline. QX-314 chloride (5 mM) was in the pipette solution for evoked recordings. All data were collected in voltage-clamp mode using a Multiclamp 700B patch-clamp amplifier (Molecular Devices, San Jose, CA, USA) and a Digidata 1500B (Axon Digidata 1500B, Molecular Devices). Asynchronous release during 20 Hz stimulation (i.e., tonic charge transfer during the train) was quantified as illustrated in [Fig. 4A](#). This was done by calculating the area between the baseline, before the first stimulus, and the tail current values, which were measured 1 ms before each stimulus in the train ([Otsu et al., 2004](#); [Yao et al., 2011](#)). All recordings and analysis were performed blind to genotype.

High-pressure freezing and freeze substitution

Cells cultured on sapphire disks were frozen using an EM ICE high-pressure freezer (Leica Microsystems), exactly as previously described ([Kusick et al., 2020](#)). The freezing apparatus was assembled on a table heated to 37 °C in a climate control box, with all solutions pre-warmed (37 °C). Sapphire disks with neurons were carefully transferred from the culture medium to a small culture dish containing physiological saline solution (140 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose; pH adjusted to 7.3 with NaOH, 300 mOsm). NBQX (3 μM; Tocris) and bicuculline (30 μM; Tocris) were added to the physiological saline solution to block recurrent synaptic activity. CaCl₂ and MgCl₂ concentrations were 1.2 mM and 3.8 mM, respectively. After freezing, samples were transferred under liquid nitrogen to an EM AFS2 freeze substitution system at -90 °C (Leica Microsystems). Using pre-cooled tweezers, samples were quickly transferred to anhydrous acetone at -90 °C. After disassembling the freezing apparatus, sapphire disks with cells were quickly moved to cryovials containing freeze substitution solutions. For the first two of three Syt7 knockout freezes, freeze substitution was performed exactly as previously described in [Kusick et al., 2020](#): solutions were 1% glutaraldehyde, 1% osmium tetroxide, and 1% water in anhydrous acetone, which had been stored under liquid nitrogen then moved to the AFS2 immediately before use. The freeze substitution program was as follows: -90 °C for 6-10 hr (adjusted so substitution

would finish in the morning), 5 °C h⁻¹ to -20 °C, 12 h at -20 °C, and 10 °C h⁻¹ to 20 °C. For all other experiments, we used a different freeze substitution protocol that yields more consistent, high-contrast morphology, as described in [Vevea et al., 2021](#) [\[1\]](#): samples were first left in 0.1% tannic acid and 1% glutaraldehyde at -90 °C for ~36 hours, then washed 5x, once every 30 min, with acetone, and transferred to 2% osmium tetroxide, then run on the following program: 11 hr at -90 °C, 5 °C h⁻¹ to -20 °C, -20 °C for 12 hr, 10 °C h⁻¹ to -4 °C, then removed from the freeze substitution chamber and warmed at room temperature for ~15 min before washing. For this latter protocol, instead of cryovials, solutions and sapphires were stored in universal sample containers (Leica Microsystems), covered in aclar film to prevent evaporation. Using these containers instead of cryovials makes all solution transfers, from fixation through infiltration and embedding, much faster and more convenient.

Infiltration, embedding, sectioning, and transmission electron microscopy

Infiltration, embedding, and sectioning were performed as previously described ([Kusick et al., 2020](#) [\[2\]](#)), except without en-bloc staining, which we found to not improve morphology. Samples in fixatives were washed three times, 10 min each, with anhydrous acetone, then left in 30% epon araldite (epon, 6.2 g; araldite, 4.4 g; DDSA, 12.2 g; and BDMA, 0.8 ml, diluted in acetone) for 3 h, then 70% epon araldite for 2 h, both with shaking. Samples were then transferred to caps of polyethylene BEEM capsules (EMS) and left in 90% epon araldite overnight at 4 °C. The next morning, samples were transferred to 100% epon araldite for 1 h, then again to 100% for 1 h, and finally transferred to 100% epon araldite and baked at 60 °C for 48 h. For ultramicrotomy, 40-nm sections were cut. Sections on single-slot grids coated with 0.7% pioloform were stained with 2.5% uranyl acetate then imaged either at 120 kV on the 57,000x setting on a Thermo-Fisher Talos L120C equipped with a Ceta camera (pixel size = 244 pm; first replicate of the Doc2 experiment) or at 80 kV on the 100,000x setting on a Hitachi 7600 TEM equipped with an AMT XR50 camera run on AMT Capture v6 (pixel size = 560 pm, all other experiments). Samples were blinded before imaging. To further limit bias, synapses were found by bidirectional raster scanning along the section at the magnification at which images were taken, which makes it difficult to “pick” certain synapses, as a single synapse takes up most of this field of view. Synapses were identified by a vesicle-filled presynaptic bouton and a postsynaptic density. Postsynaptic densities are often subtle in our samples, but synaptic clefts were also identifiable by 1) their characteristic width, 2) the apposed membranes following each other closely, and 3) vesicles near the presynaptic active zone. 125-150 micrographs per sample of anything that appeared to be a synapse were taken without close examination. All images were from different synapses (that is, images were never captured from different sections of the same synapse). Most of the time all images for a condition were taken from a single section, each of which typically contains hundreds of different boutons; when different sections were used, they were always at least 10 serial sections apart (400 nm in z) to ensure they didn’t contain any of the same boutons.

Electron microscopy image analysis

EM image analysis was performed as previously described ([Kusick et al., 2020](#) [\[2\]](#)). All the images from a single experiment were randomized for analysis as a single pool. Only after this randomization were any images excluded from analysis, either because they appeared to not contain a bona fide synapse, or the morphology was too poor for reliable annotation. The plasma membrane, the active zone, docked synaptic vesicles, and all synaptic vesicles within ~100 nm of the active zone were annotated in ImageJ using SynapsEM plugins ([Watanabe et al., 2020](#) [\[3\]](#)) [<https://github.com/shigekiwatanabe/SynapsEM> [\[3\]](#) copy archived at [swh:1:rev:11a6227cd5951bf5e077cb9b3220553b506eadbe](https://www.swh.io/rev/11a6227cd5951bf5e077cb9b3220553b506eadbe)]. The active zone was identified as the region of the presynaptic PM with the features described above for identifying a synapse. Docked vesicles were identified by their membrane appearing to be in contact with the PM at the active zone (0 nm from the PM); that is, there are no lighter pixels between the membranes. Vesicles that were not manually annotated as docked but were 0 nm away from the active zone PM were automatically counted as

docked when segmentation was quantitated (see below) for data sets counting the number of docked vesicles. Likewise, vesicles annotated as docked were automatically placed in the 0 nm bin of vesicle distances from the PM. To minimize bias and error, and to maintain consistency, all image segmentation, still in the form of randomized files, was thoroughly checked and edited by a second member of the lab. Features were then quantitated using the SynapsEM family of MATLAB (MathWorks) scripts (<https://github.com/shigekiwatanabe/SynapsEM>). Example electron micrographs shown were adjusted in brightness and contrast to different degrees (depending on the varying brightness and contrast of the raw images), rotated, and cropped in ImageJ before import into Adobe Illustrator.

Statistics

Statistical analysis was performed using GraphPad Prism 9 software. iGluSnFR and electrophysiology data were presented as mean $\pm$ standard error of the mean. Electron microscopy data were presented as mean and 95% confidence interval of the mean. For iGluSnFR and electrophysiology, data sets were tested for normality using the Anderson-Darling test and corresponding parametric or non-parametric hypothesis tests performed, as stated in the figure legends. Since electron microscopy pit and docked vesicle data are count data, following a roughly binomial rather than normal distribution, means were used to graph the data (medians are not a useful representation of central tendency for count data, particularly low-frequency count data; for example, the median number of pits in all samples is 0 since exocytic pits are rare) and Games-Howell post-hoc test used, which does not assume a normal distribution.

Mathematical model

In the model we developed, a release site can be in one of three states: empty, occupied with a tethered vesicle or occupied with a docked vesicle (for full scheme see Fig. S9). Only docked vesicles can fuse and their fusion rates depend on the number of Ca^{2+} ions bound to syt1 and Doc2 α . Ca^{2+} binding to these Ca^{2+} sensors followed the dual-sensor scheme as previously published (Kobbersmed et al., 2020; Sun et al., 2007), in which five Ca^{2+} ions per vesicle can bind to the first Ca^{2+} sensor for fusion (syt1 in our model), and two Ca^{2+} ions to the second Ca^{2+} sensor for fusion (Doc2 α in our model). We adjusted the dual-sensor model such that no more than five Ca^{2+} ions can be bound in total to syt1 and Doc2 α simultaneously. This is based on the idea that syt1 and Doc2 α are competing for the same resources, which could for instance be a limited number of SNARE complexes that are available to execute the fusion reaction. Ca^{2+} can bind to Doc2 α at any of the release site states, whereas Ca^{2+} binding to syt1 only occurs when vesicles are docked. This is because Doc2 α is assumed to reside on the release site (Verhage et al., 1997) and syt1 is attached to the synaptic vesicle (Takamori et al., 2006). Together this means that an empty release site can be in three different states that are described by the following differential equations:

$$\begin{aligned}
 \frac{dE[0]}{dt} &= -k_{tet}E[0] + k_{untet}T[0] + \sum_{i=0}^5 (f(0,i)D[0,i]) + k_{2-}E[1] - 2[Ca^{2+}]k_{2+}E[0] \\
 \frac{dE[1]}{dt} &= -k_{tet}E[1] + k_{untet}T[1] + \sum_{i=0}^5 (f(1,i)D[1,i]) + 2k_{2-}b_2E[2] \\
 &\quad + 2[Ca^{2+}]k_{2+}E[0] - (Ca^{2+}k_{2+} + k_{2-})E[1] \\
 \frac{dE[2]}{dt} &= -k_{tet}E[2] + k_{untet}T[2] + \sum_{i=0}^5 (f(2,i)D[2,i]) + [Ca^{2+}]k_{2+}E[1] - 2k_{2-}b_2E[2]
 \end{aligned}
 \tag{1}$$

In these equations $E[d2]$, $T[d2]$, $D[d2, s1]$ denotes the Ca^{2+} binding state of the empty release, tethered vesicle and docked vesicle, respectively, with $d2$ the number of Ca^{2+} ions bound to Doc2 α and $s1$ the number of Ca^{2+} ions bound to syt1 per release site. f is the fusion rate function that is described in [equation 5](#). A definition of the model parameters indicated in the equations can be found in supplementary table 3. A release site occupied with a tethered vesicle can be in three different states, depending on the Ca^{2+} -binding state of Doc2 α .

$$\begin{aligned} d \frac{T[0]}{dt} &= k_{tet} E[0] - k_{untet} T[0] + \sum_{i=0}^5 (undocking D[0, i]) - docking T[0] + k_{2-} T[1] \\ &\quad - 2[Ca^{2+}] k_{2+} T[0] \\ d \frac{T[1]}{dt} &= k_{tet} E[1] - k_{untet} T[1] + \sum_{i=0}^5 (undocking D[1, i]) - docking T[1] + 2k_{2-} b_2 T[2] \\ &\quad + 2[Ca^{2+}] k_{2+} T[0] - ([Ca^{2+}] k_{2+} + k_{2-}) T[1] \\ d \frac{T[2]}{dt} &= k_{tet} E[2] - k_{untet} T[2] + \sum_{i=0}^5 (undocking D[2, i]) - docking T[2] \\ &\quad + [Ca^{2+}] k_{2+} T[1] - 2k_{2-} b_2 T[2] \end{aligned} \quad (2)$$

Undocking and docking rates are calculated based on the equations below ([equations 6](#) and [7](#)). The following equation describes the Ca^{2+} bound state of docked vesicles:

$$\begin{aligned} d \frac{D[d2, s1]}{dt} &= \mathbf{1}_{s1 \geq 1} \max(0, 5 - (d2 + s1 - 1)) [Ca^{2+}] k_{1+} D[d2, s1 - 1] \\ &\quad + \mathbf{1}_{s1 < 5} (s1 + 1) b_1^{s1} k_{1-} D[d2, s1 + 1] \\ &\quad + \mathbf{1}_{d2 \geq 1} \min(\max(0, 2 - d2 + 1), \max(0, 5 - (d2 + s1 - 1))) [Ca^{2+}] k_{2+} D[d2 - 1, s1] \\ &\quad + \mathbf{1}_{d2 < 2} (d2 + 1) b_2^{d2} k_{2-} D[d2 + 1, s1] \\ &\quad + \mathbf{1}_{s1 = 0} docking T[d2] \\ &\quad - (\max(0, 5 - (d2 + s1)) [Ca^{2+}] k_{1+} + s1 b_1^{s1} k_{1-} + \min(\max(0, 2 - d2), \max(0, 5 - (d2 + s2))) [Ca^{2+}] k_{2+} + d2 b_2^{d2-1} k_{2-} + f(d2, s1) + undocking) D[d2, s1] \end{aligned} \quad (3)$$

Fusion can occur from either of the docked states with f being the fusion rate function (see [equation 5](#)). The change in the cumulative number of fused vesicles (F) is described by:

$$\frac{dF}{dt} = f(d2, s1) D[d2, s1] \quad (4)$$

where f is the fusion rate function:

$$f(d2, s1) = l_+ f_1^{s1} f_2^{d2} \quad (5)$$

f_1 and f_2 are the factors by which each Ca^{2+} ion bound to either syt1 ($s1$) or Doc2 α ($d2$) increases the release rates of a synaptic vesicle, and l_+ equals the basal fusion rate (supplementary table 3). In the model docking and undocking rates are dependent on the number of Ca^{2+} ions bound to syt7 ($s7$). Binding of Ca^{2+} to all syt7s expressed per release site follows the same scheme as Doc2 α , and can be described by the following rate equations:

$$\begin{aligned} d \frac{S[0]}{dt} &= k_{7-} S[1] - (2 k_{7+} [Ca^{2+}]) S[0] \\ d \frac{S[1]}{dt} &= 2 [Ca^{2+}] k_{7+} S[0] + 2 b_7 k_{7-} S[2] - (k_{7+} [Ca^{2+}] + k_{7-}) S[1] \\ d \frac{S[2]}{dt} &= [Ca^{2+}] k_{7+} S[1] - (2 b_7 k_{7-}) S[2] \end{aligned} \quad (6)$$

Here, $S[s7]$ denotes the proportion of release sites with $s7$ number of Ca^{2+} ions bound to syt7. We assumed that syt7 bound to Ca^{2+} functions as a catalyst for docking, and thus increases both docking and undocking rates. In this way, all proteins included in the model function by lowering an energy barrier (for docking for syt7 and for fusion for Doc2a and syt1/2). From these states the docking and undocking rates can be computed following:

$$\begin{aligned} \text{docking} &= k_{\text{docking}} \sum_{s7=0}^2 S[s7] f_7^{s7} \\ \text{undocking} &= k_{\text{undocking}} \sum_{s7=0}^2 S[s7] f_7^{s7} \quad (7) \end{aligned}$$

In these equations f_7 is the factor describing how much an individual Ca^{2+} ion bound to syt7 increases the rates of vesicle docking and undocking.

To compute the steady state of the system, all reaction rates (from one state to another, except the Ca^{2+} binding states of syt7) were rewritten into an intensity matrix, Q , in which element i,j describes the rate of transitioning from state i to state j . Subsequently, steady state was computed using the equation:

$$\pi_{ss} = \pi_i e^{Qt} \quad (8)$$

With π_i a vector of 24 zeros, corresponding to each of the release site states (independent of syt7). The first element of this vector was set to total number of release sites in the model. t was set to 1000s. This large evaluation time was used to ensure that steady state was reached. The resulting π_{ss} corresponds to the distribution of the different release site states described in the [equations 1–3](#) at steady state. The steady state of syt7 was solved separately by setting the syt7 unbound state ($S[0]$) to 1. Subsequently $S[1]$ and $S[2]$ were computed by:

$$\begin{aligned} S[1] &= \frac{2[\text{Ca}^{2+}]k_{7+}}{k_{7-}} \\ S[2] &= \frac{[\text{Ca}^{2+}]k_{7+}}{b_7 k_{7-}} \quad (9) \end{aligned}$$

Next, $S[0]$, $S[1]$ and $S[2]$ were divided by the sum of those three elements, to obtain a distribution of the different syt7 bound states at steady state. The steady state of the system was computed for a resting Ca^{2+} concentration of 0.05 μM ([Helmchen et al., 1997](#)).

With the steady state calculation as a starting point, the differential equations were solved in Matlab (2020b) using ode15s (MaxStep = $1e^{-5}$, RelTol = $1e^{-5}$, AbsTol = $1e^{-5}$) and a Ca^{2+} transient corresponding to ten APs at 20Hz as input. The Ca^{2+} -transient for a single AP was generated using a normal distribution (mean = +1.5ms, sd = 0.2ms), which was scaled to obtain the desired amplitude (supplementary table 3). To this Ca^{2+} -transient a residual signal was added, which followed an exponential decay:

$$\text{Ca}_{\text{residual}}^{2+}(t) = \mathbf{1}_{t \geq t_0} A e^{-\frac{(t-t_0)}{\tau}} \frac{t-t_0}{t-t_0+km} \quad (10)$$

with, t_0 the starting times of the residual Ca^{2+} -signal (+1.6 ms from the onset of the AP), τ the decay time constant, t time, and A the amplitude of the residual Ca^{2+} signal. The second term in the equation $\frac{t-t_0}{t-t_0+km}$ ensures smoothing of the Ca^{2+} -transient. In this term, km was set to 0.1ms. To the entire Ca^{2+} -transient, a basal concentration of 0.05 μM was added ([Helmchen et al., 1997](#)).

The simulated cumulative number of fused vesicles (F , in equations 4) was interpolated at a sampling rate of $50e^3$ Hz. From this release rates were computed by multiplying the difference between two time points with the sampling rate. The maximum release rate between stimulus

onset (0ms) and the onset of the next stimulus (+50ms) was taken as the peak release rate of each stimulus. All release events occurring at +5ms from stimulus onset to the onset of the next stimulus (+50ms) were considered as asynchronous release events.

Parameters in the model were set to experimentally obtained values, if available. For a list of all model parameters see supplementary table 3. To simplify the model, we assumed that both syt7 and Doc2 α have the same Ca²⁺-binding dynamics, as the scope of the model was to investigate the apparent role of syt7 and Doc2 α in asynchronous release when they act on different steps in the reaction scheme. Furthermore, undocking and untethering rate constants were set equal to the docking and tethering rate constants, respectively. This assumption was made to reduce the number of free parameters in the model. The remaining free parameters, f_2 , f_7 , k_{tet} , k_{docking} , amplitude of Ca²⁺-transient, amplitude of residual Ca²⁺ (A) and decay time of residual Ca²⁺ signal (τ), were adjusted manually to obtain close correspondence between model predictions and the phenotypes observed in the experiments presented in this manuscript. More specifically, we decided whether the model could recapitulate experimental observations based on the number of vesicles docking between 5 and 15 ms after an AP (this should be as large as possible for the WT and Doc2 α -model, and almost 0 for the syt7-), the peak release rate in response to the first AP (to be equal between all conditions), the ratio between the peak release rate of the 1st and 10th response (depressive phenotype should be the most prominent in syt7- and the least prominent in Doc2 α -) and the amount of asynchronous release (syt7- and Doc2 α -should have approximately half of the total amount of asynchronously released vesicles compared to control). Moreover, the parameter values of the Ca²⁺ transient should be realistic. We do not know the exact values of the Ca²⁺ transient for the hippocampal samples used in this study, but multiple previous studies have provided a range of realistic parameter values (Brenowitz and Regehr, 2007 [↗](#); Helmchen et al., 1997 [↗](#); Sabatini and Regehr, 1998 [↗](#); Wang et al., 2008 [↗](#)). For

simulations of the model lacking Doc2 α , k_{2+} was set to 0. To simulate syt7-conditions, f_7 was set to 1. The effect of each model parameter on the quantified behavior of the model (number of vesicles docking between 5ms and 15ms, total asynchronously released vesicles at the end of the train, peak release rate of the first response, and the ratio between peak release rate of the first and the tenth response) was determined on a range from one-tenth of the selected parameter value to ten-fold the selected parameter value (as in supplementary table 3).

Data availability

Full data tables underlying all figures are available in the source data. Raw data and analysis files are available upon request.

Code availability

Custom R, Matlab and Fiji scripts for electron microscopy analysis are available at <https://github.com/shigekiwatanabe/SynapsEM> [↗](#). More details can be found in Watanabe et al., 2020 [↗](#). All code for the mathematical model is available with the paper.

Figure legends

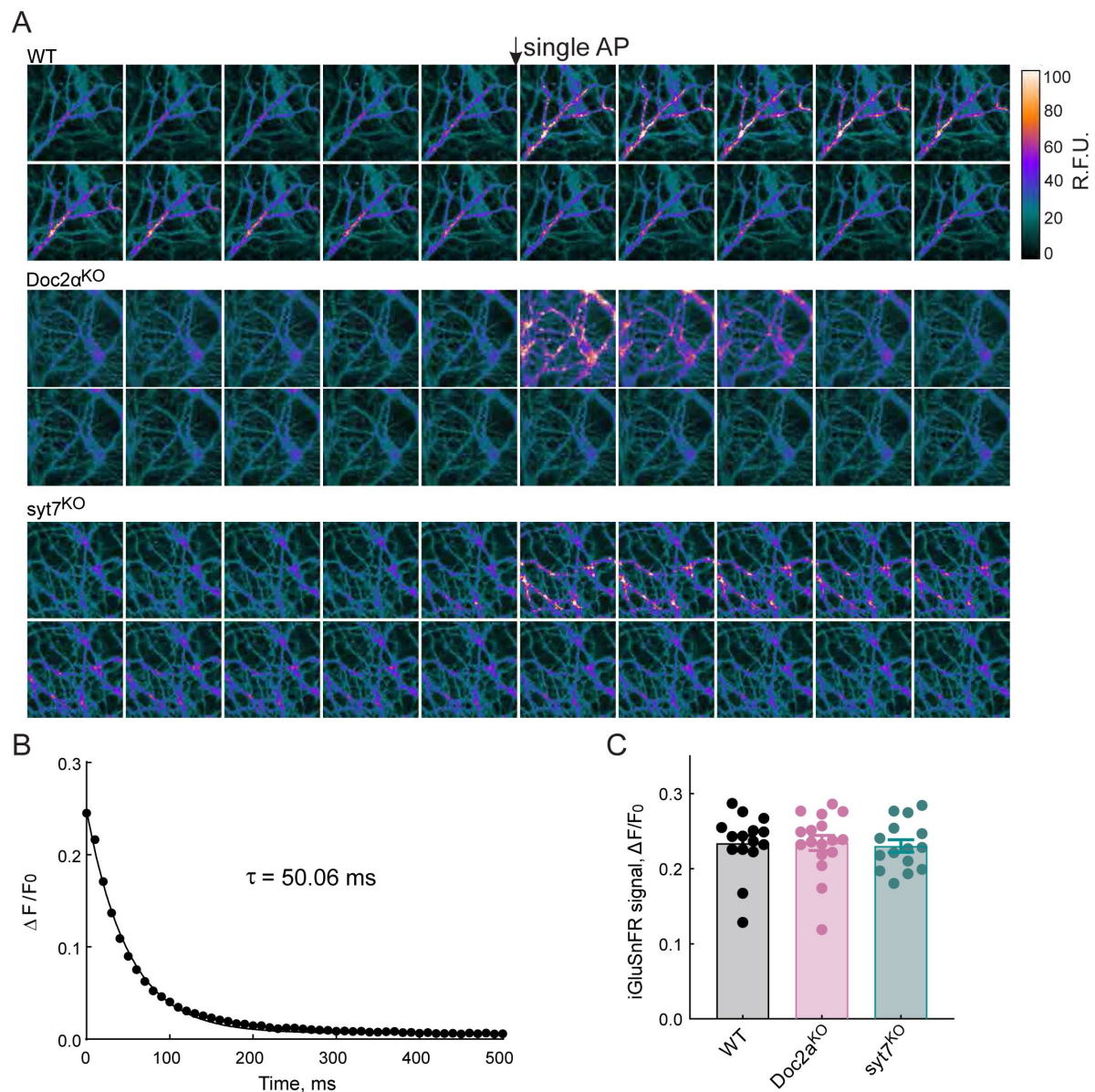


Fig. S1.

Expression of iGluSnFR in cultured hippocampal neurons.

(A) Representative serial frames of iGluSnFR fluorescence from the WT, Doc2a^{KO}, and syt7^{KO} neurons, before and after eliciting a single action potential (AP; arrow); the heatmap is shown on the right.

(B) The relaxation rate of iGluSnFR (A184V). Averaged iGluSnFR signals from 254 boutons from WT hippocampal neurons after a single stimulus (filled circles). Data were fitted with a single exponential function (solid line), and the decay τ value is indicated.

(C) iGluSnFR peak amplitudes ($\Delta F/F_0$ at 10 ms) after a single action potential, from WT, Doc2a KO, and syt7 KO neurons.

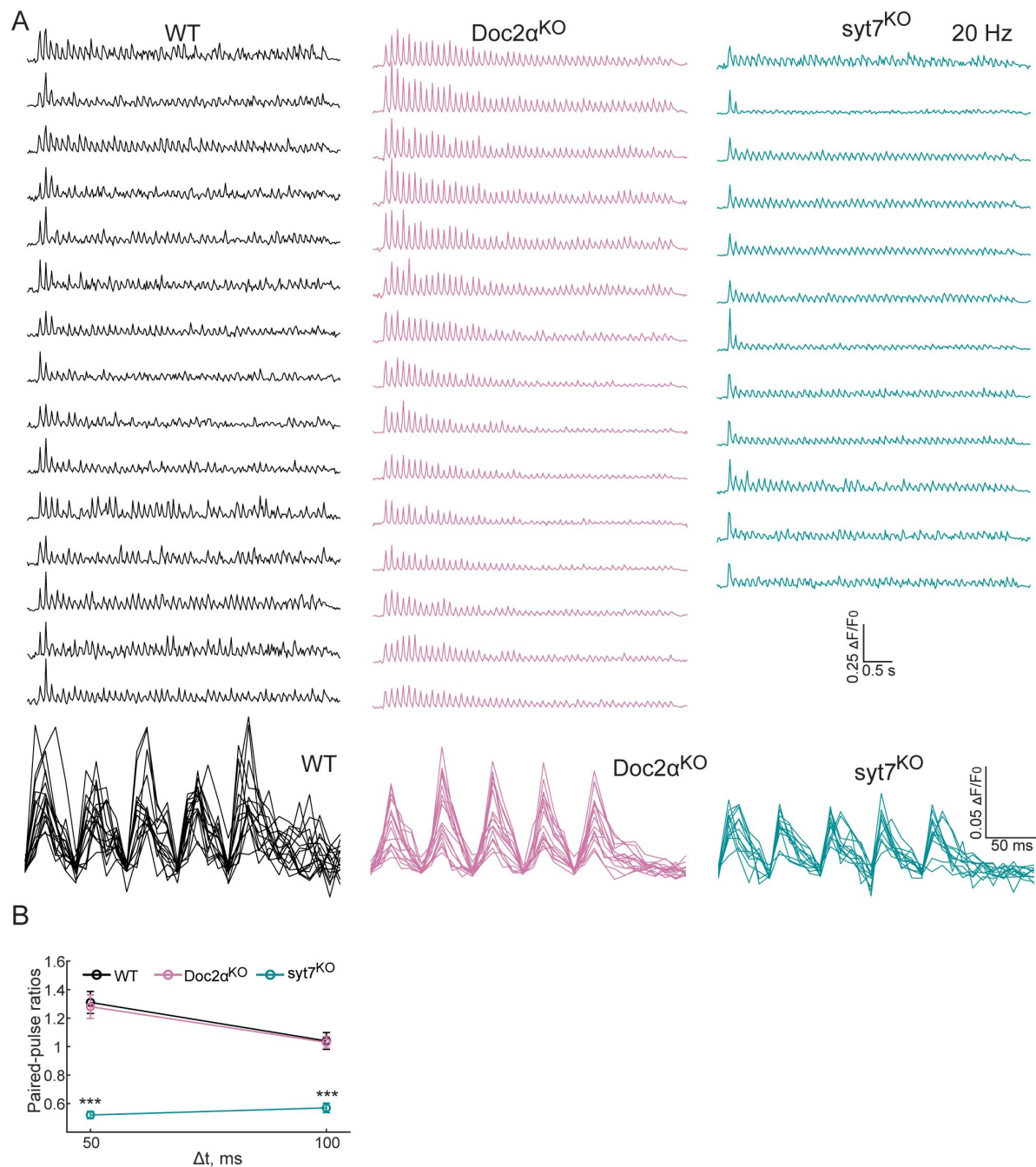
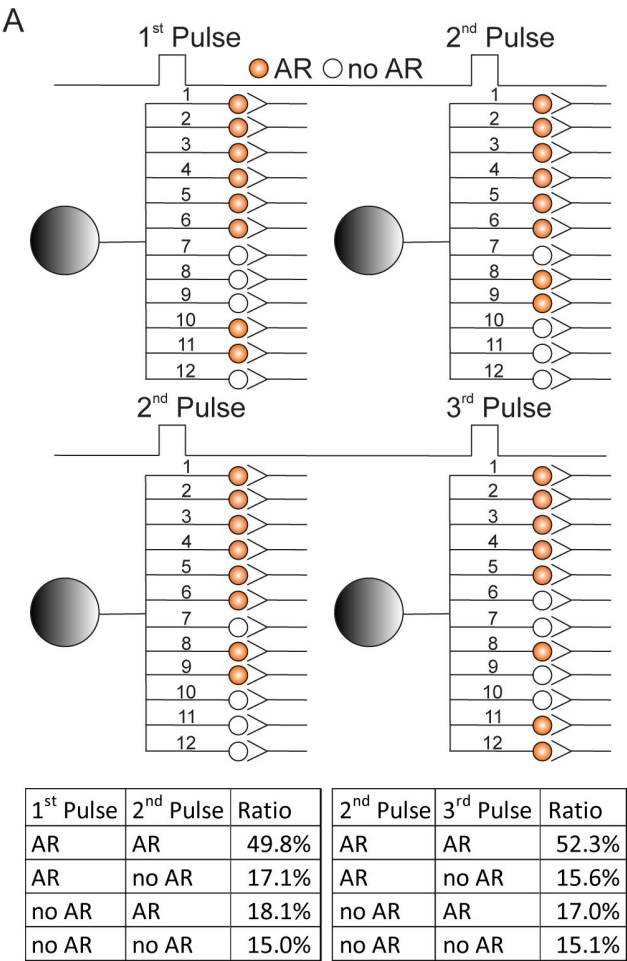


Fig. S2.

Additional raw iGluSnFR traces during 20 Hz train stimulation, and paired-pulse ratios from the first two responses at 10 and 20 Hz.

(A) Raw 20 Hz iGluSnFR traces from cultured WT, Doc2α^{KO}, and syt7^{KO} hippocampal neurons. In the lower panel, responses from the last five stimuli are overlaid, revealing a qualitative reduction in AR peaks in Doc2α and syt7 KO neurons.

(B) Mean paired-pulse ratios (peak iGluSnFR signal from the 2nd stimulus divided by peak signal from the 1st stimulus during the 10Hz and 20 Hz train stimulation) at different interstimulus intervals (Δt) recorded in cultured hippocampal neurons isolated from WT, Doc2α^{KO}, and syt7^{KO} mice. Data points here are mean values $\pm$ SEM. One-way ANOVA, followed by Dunnett's test, was used to compare mutants against WT. *** indicates $p < 0.001$.



B

For all three of the first stimuli in a 10 Hz train:		
Responses	Percent	No. of boutons out of 923
AR only	5.53	51
SR only	8.45	78
AR	40.4	373

Fig. S3.

Spatial distribution of AR, measured via iGluSnFR imaging, in cultured hippocampal neurons.

Schematic illustrating AR distribution patterns at axon terminals; the large shaded grey circles depict the soma; small circles depict presynaptic boutons. For this analysis, only AR was analyzed, using data collected from 923 ROIs that were initially identified because they yielded at least one evoked response (AR, synchronous, or both) in the first three stimuli from a 10 Hz train. Boutons that exhibit AR, for the indicated position and stimulus, are orange; open circles indicate boutons that did not exhibit AR at the indicated position or stimulus. All of the AR distribution patterns in each of the four sub-panels are from the same boutons at the same fixed positions. For convenience, only twelve boutons are shown, and these are numbered 1-12 to emphasize that the same boutons are analyzed in each of the sub-panels. The organization of these boutons was artificially grouped to some extent, to better visualize the patterns of release between each stimulus. The bottom left table quantifies the ratio of AR patterns: AR to AR, AR to no AR, no AR to AR, and no AR to no AR, from the first to the second stimulus. The same analysis, but between the second and third stimulus, is provided in the bottom right table. The distribution of AR to target cells in the first versus the second pulses is similar to the distribution between the third and fourth pulses. These data suggest that AR during the first pulse, while molecularly distinct, does not segregate to different target cells, as compared to AR elicited by subsequent stimuli.

(A) Table summarizing the fraction of boutons with the same responses after the initial three stimuli. AR only; represents pure AR that successively occurred three times at the same bouton, in the absence of SR. Synchronous release (SR) only; indicates boutons that exhibited purely SR during the first three stimuli, in the absence of AR. AR; reflects AR that occurred at the same bouton for all three successive stimuli regardless of the presence or absence of SR. Interpretation: In the main text we noted that spatial segregation of AR during the first two responses was not observed. Expanding on this further, the same pattern was observed when comparing the second and third responses. Moreover, nearly 70% of all responding boutons exhibited AR at least once during the first three stimuli in a train, either with or without a concurrent synchronous component (panel 2A). So, even though AR presents only a third of the charge transfer in our experiments (Fig. 1C,E and 2D), this mode of release is widespread. In addition, 40% of all responding boutons analyzed exhibited AR in all three of the initial stimuli in a train, and approximately half of the boutons that had AR after the first stimulus also yielded AR during the second stimulus (panel 2B). In contrast, only 6% and 8% of boutons exhibited pure AR or synchronous responses, respectively, in response to all three initial stimuli in a train (panel 2B). While our data argue against the segregation of AR during the first response, it remains possible that further analysis, of much larger data sets, using sensitive algorithms, could reveal more subtle differences that might have escaped detection.

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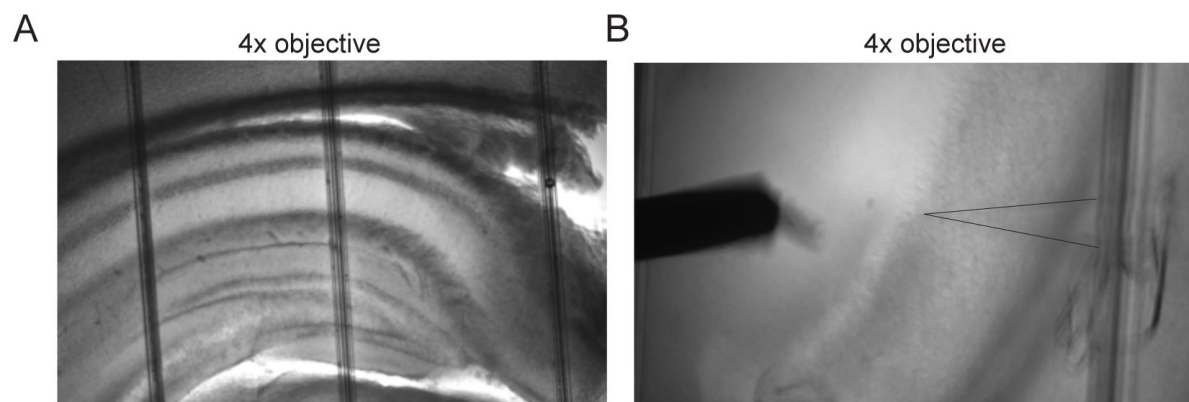


Fig. S4.

AR in acute brain slices from Doc2 α and syt7 KO mice during 10 Hz train stimulation.

(A) Representative hippocampal slice from a WT mouse.

(B) A stimulating electrode was placed in the Schaffer collateral pathway and transmission to CA1 neurons was monitored via whole-cell patch clamp recordings.

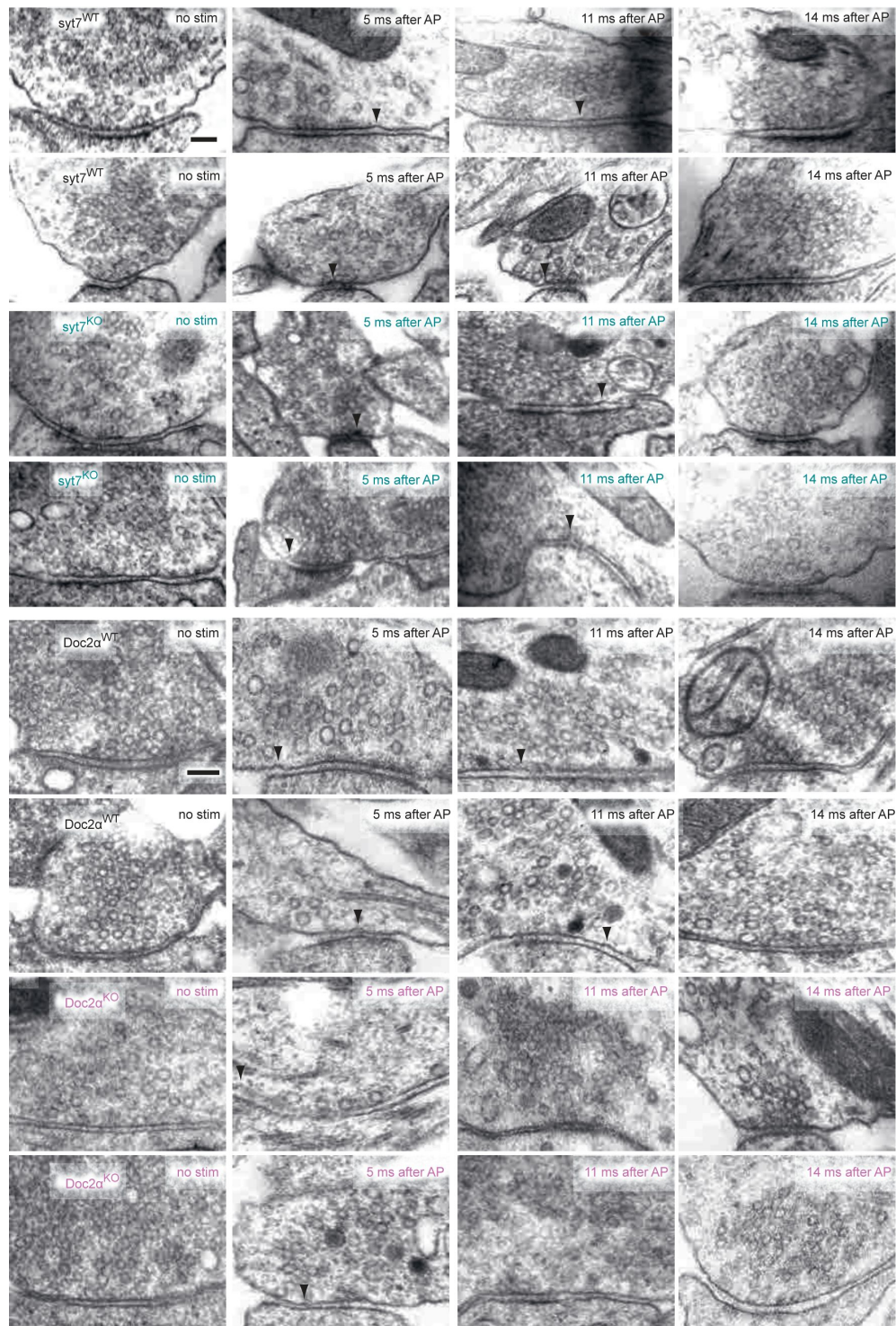


Fig. S5.

Additional example transmission electron micrographs.

From the experiments described in [Fig. 5](#) and [Fig. 6](#). Scale bar: 100 nm.

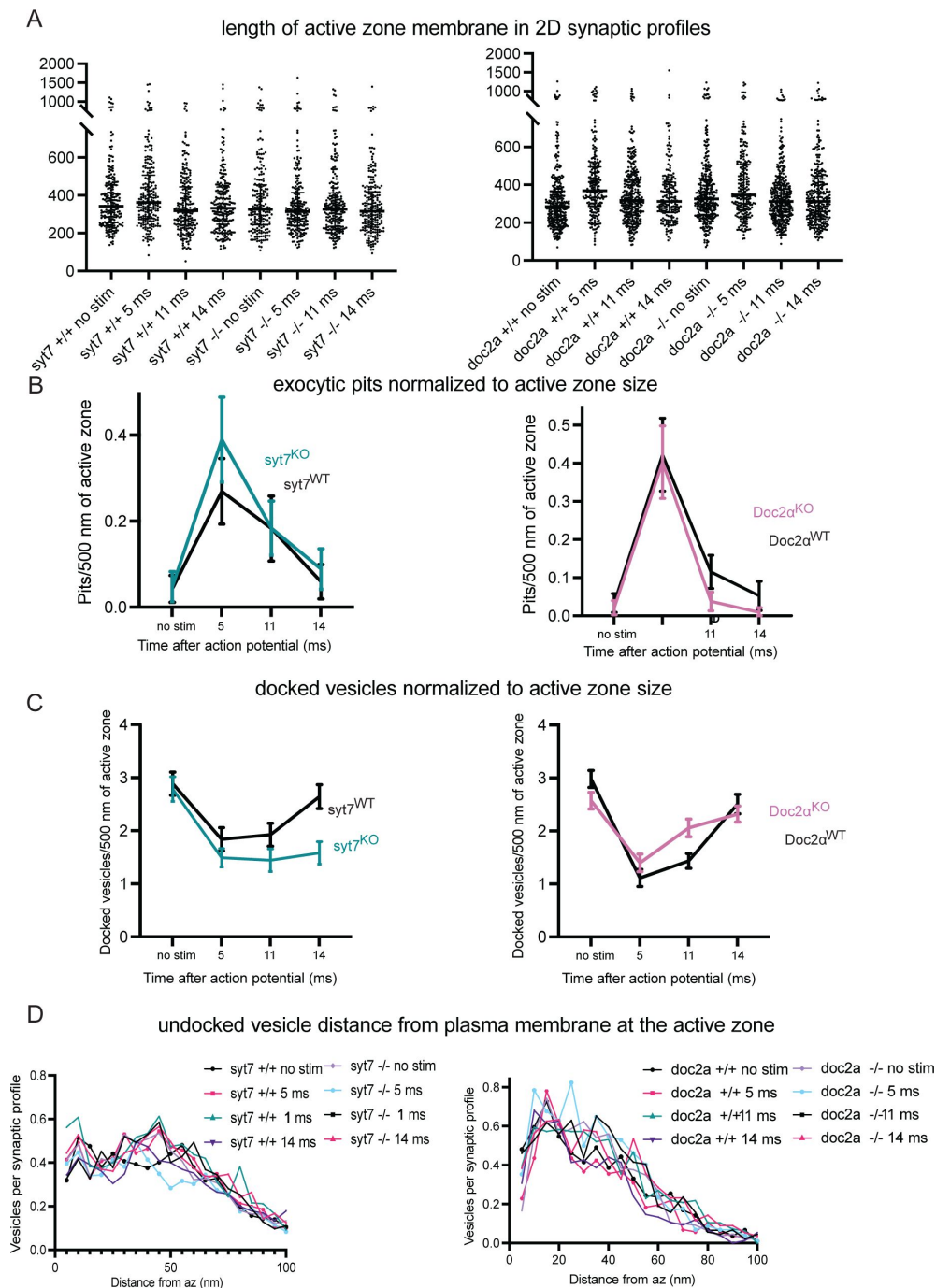


Fig. S6.

Additional quantification from electron microscopy.

From the experiments described in [Fig. 5](#) and [Fig. 6](#).

(A) Size of the active zone in each synaptic profile from each condition. Each dot indicates a single synaptic profile. Error bars: median and interquartile range. Same pit data from [Fig. 5](#), normalized to the size of the active zone in each condition. "Per 500 nm of active zone" calculation was performed by multiplying the count from each synaptic profile by 500 and dividing by the mean active zone size from the data set (i.e., normalization was not performed by the size of the active zone in each individual synaptic profile).

(B) Same as B, but for docked vesicle data from [Fig. 6](#).

(C) Distances of undocked synaptic vesicles from the plasma membrane at the active zone, not including docked vesicles (which are 0 nm from the plasma membrane). Distances are binned in 5-nm increments ("5" indicates vesicles 0.1–5 nm from the membrane, "10" indicates 6–10 nm, and so on). Each dot indicates the mean number of synaptic vesicles per synaptic profile in that distance range from the active zones.

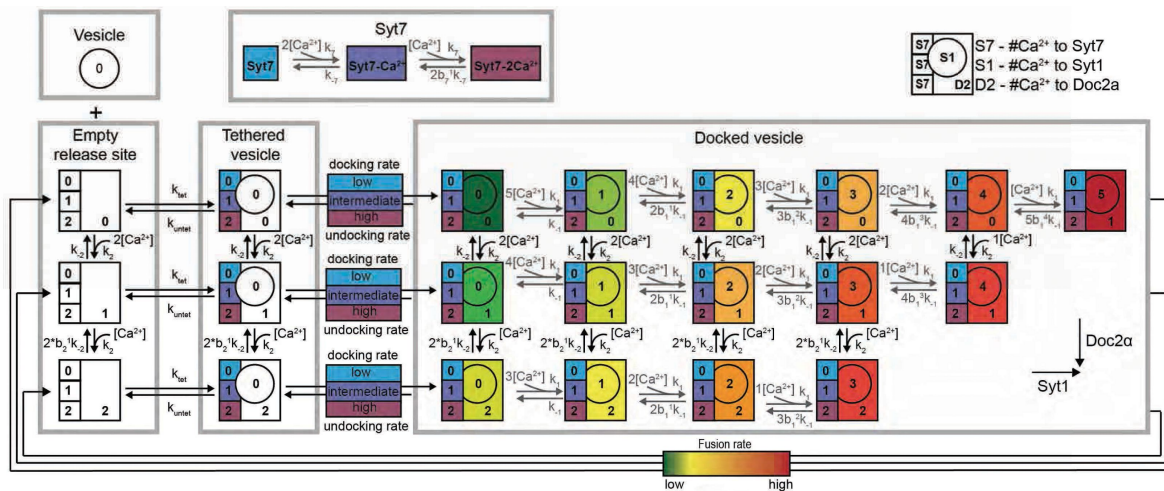


Fig. S7.

Full reaction scheme of the model

In the model a release site can be in three different states: empty, occupied with a tethered vesicle or a docked vesicle. Within those states a release site can be in a different substate depending on the number of Ca^{2+} ions bound to Doc2α, syt7 and syt1. The top left illustration shows the legend for these substates. Doc2α and syt7 are assumed to be part of the release sites. Therefore, syt7 and Doc2α can bind to Ca^{2+} in all the states. Syt1 can only bind to Ca^{2+} once the vesicle is docked. Binding of Ca^{2+} to syt7 increases both the docking and undocking rates.

Synaptic vesicle docking occurs with a basal docking rates (k_{docking}) from tethered vesicles labeled with blue, which are those release sites in which syt7 has not bound to Ca^{2+} . Purple and magenta colors indicate states with increased docking rates correspondingly tethered vesicles in which one and two Ca^{2+} ions have bound to syt7 respectively. Similarly, undocking rates from all docked vesicle states is dependent on the number of Ca^{2+} ions bound to syt7. Ca^{2+} binding to Doc2α and syt1 increases the fusion probability of the docked vesicles (green to red color coding of the different synaptic vesicle states for low to high fusion rates, respectively). For full description of the model see **Methods**.

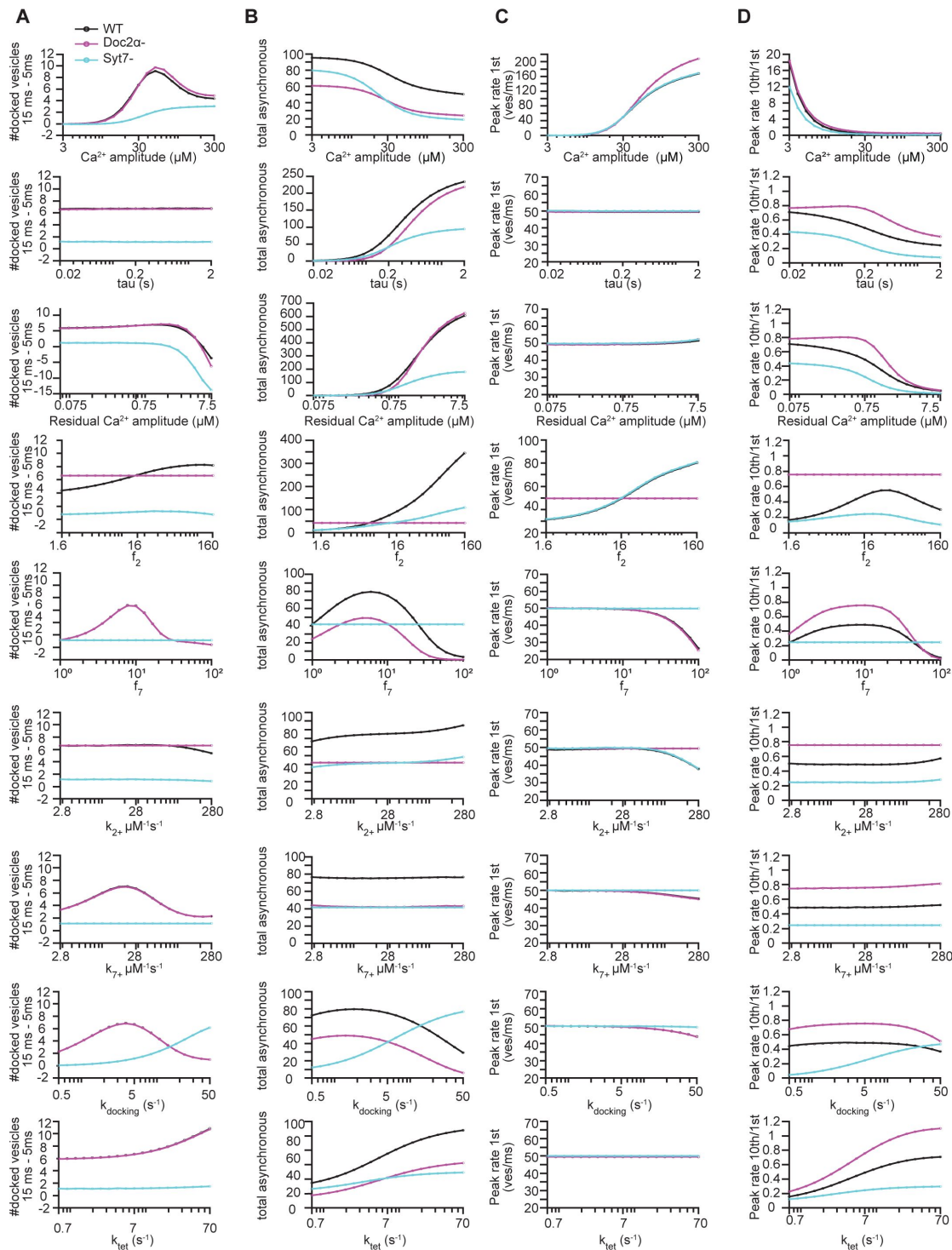


Fig. S8.

Effect of different model parameters on model predictions.

(A) Effect of varying model parameters on the number of vesicles docking between 15 and 5ms.

(B) Effect of varying model parameters on total number of asynchronously released vesicles.

(C) The effect of varying model parameters on the peak release rate at the first pulse.

(D) The effect of varying model parameters on the ratio between the peak release rates of the tenth and the first pulse.

Each parameter was evaluated at 19 values ranging from one-tenth of the selected value (as presented in supplementary table 3) to ten-fold the selected parameter value. Model parameters were varied for WT (black), Doc2 α - (magenta) and syt7- (cyan) simulations. For Ca^{2+} -binding rates (k_{2+} and k_{7+}), the unbinding rates were adjusted accordingly to have a constant Ca^{2+} -affinity. Similarly, the untethering and undocking rates were adjusted with the tethering and docking.

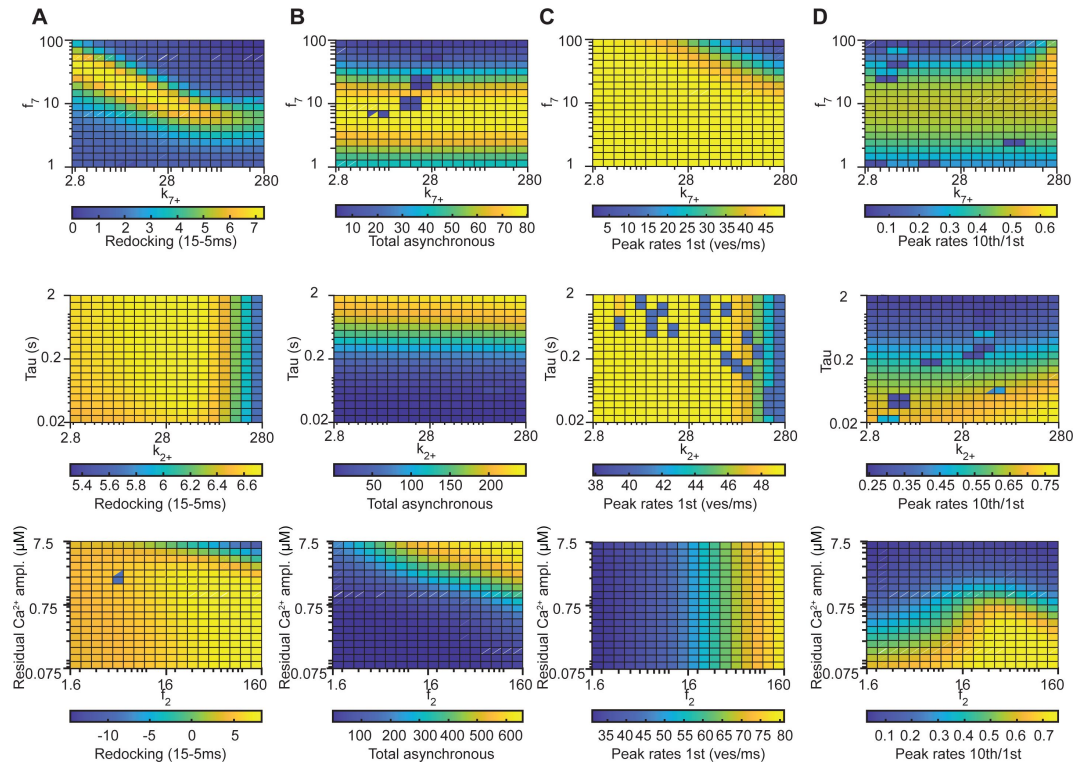


Fig. S9.

Covarying two parameters illustrates that the selected model parameters are unlikely to provide a unique solution.

(A) Effect of covarying two model parameters on the number of vesicles docking between 15 and 5ms.
 (B) Effect of covarying two model parameters on total number of asynchronously released vesicles.
 (C) The effect of covarying two model parameters on the peak release rate at the first pulse.
 (D) The effect of covarying two model parameters on the ratio between the peak release rates of the tenth and the first pulse.
 Each parameter was evaluated at 19 values ranging from one-tenth of the selected value (as presented in supplementary table 3) to ten-fold the selected parameter value. The selected setting (represented in [Fig. 7](#)) is the middle point in each panel. Covarying the Ca^{2+} -binding rate of syt7 (k_{7+}) with the effect syt7 has on the docking and undocking rates (f_7 , top panels) shows that there is a section in the parameter space where WT simulations result in similar quantified model predictions (as indicated by the same colors in the heatmaps). This also holds for covarying the Ca^{2+} -binding rate of Doc2a (k_{2+}) with the decay time of the residual Ca^{2+} -signal (tau, middle panels) and covarying of f_2 with the amplitude of the residual Ca^{2+} -signal (bottom, panels). Together, this illustrates that the selected model parameters are unlikely to present a unique solution. For Ca^{2+} -binding rates (k_{2+} and k_{7+}), the unbinding rates were adjusted accordingly to keep a constant Ca^{2+} -affinity.

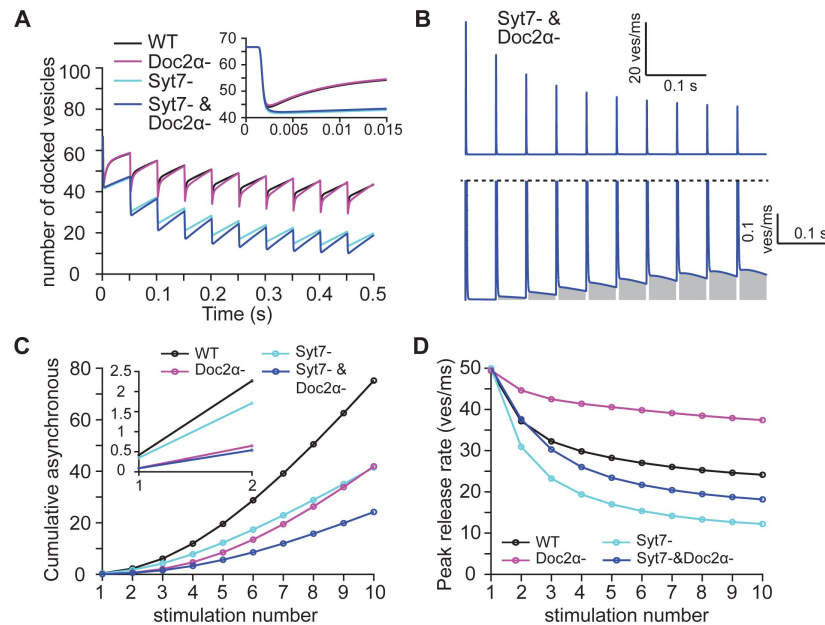


Fig. S10.

Evaluation of a model lacking both Doc2α and syt7.

(A) The number of docked vesicles over time for simulations including functionality of all proteins (black, WT), simulations lacking Doc2α (magenta, Doc2α-), simulations lacking syt7 (cyan, syt7-) and simulations lacking both syt7 and Doc2α (blue, Syt7- & Doc2α-). Insert shows a zoom-in over the first 15 ms of the simulation.

(B) Simulated release rates obtained using a model lacking syt7 and Doc2α (Syt7- & Doc2α-, blue). Top panel shows the entire trace. Bottom panel show a zoom-in to illustrate the asynchronous release component, which is indicated by the gray area.

(C) Cumulative number of asynchronously released vesicles per stimulus. Asynchronous release is quantified as the release between 5 ms after the start of the AP and the start of the next AP. Insert shows zoom-in over the first 2 pulses.

(D) Peak release rates per stimulus for stimulations using the full model (black, WT), simulations lacking Doc2α (magenta, Doc2α-), simulations lacking syt7 (cyan, syt7-) and simulations lacking both syt7 and Doc2α (blue, Syt7- & Doc2α-).

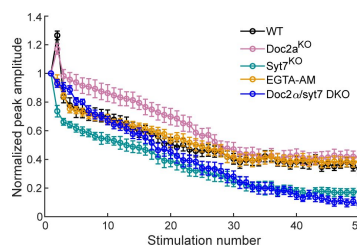


Fig. S11.

Synaptic depression of phasic responses in Doc2α/syt7 DKO, compared to single KO and AM-EGTA treated conditions.

Recordings of CA1 neurons in hippocampal slices were carried out as described in Fig. 4. The peak amplitude of each EPSC during 50 APs at 20Hz was normalized to the first EPSC from Doc2α/syt7 DKO slices; for comparison, all other conditions were replotted from Fig. 4.

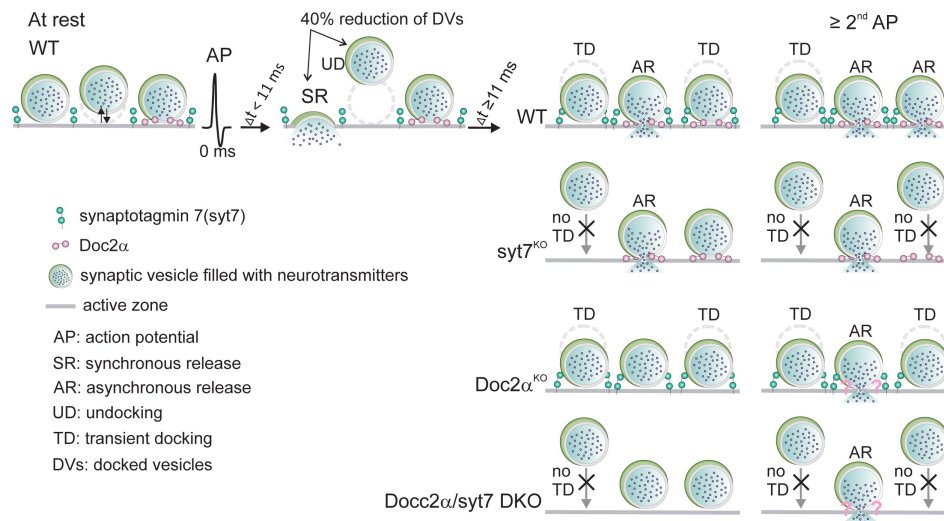


Fig. S12.

Doc2α-triggers AR in response to single action potentials; during repetitive stimulation, syt7 mediates transient docking to feed docked vesicles to Doc2α for ongoing AR.

At rest, synaptic vesicles are docked at the active zone (solid gray line) in a dynamic equilibrium indicated by the antiparallel black arrows (far left panel). Under these conditions, docking is not affected by syt7 (Fig. 6E; Vevea et al., 2021). A single action potential triggers both undocking (UD; Kusick et al., 2020) and synchronous release (SR), resulting in a 40% reduction in docked vesicles in ~5 ms (Fig. 6E,F; Kusick et al., 2020). This is followed by a transient docking (TD) step in which vesicles re-attach to the active zone via a process that takes ~14 msec and is mediated by Ca²⁺ and syt7 (granite green) (Fig. 6E,F; Kusick et al., 2020). The same single AP also triggers AR mediated directly by Doc2α (lilac), at the 11 ms time point (Fig. 5F). Syt7 plays no role in release during the first action potential (Fig. 1, 2, and 5E; and references in the main text), again because it does not regulate docking in the resting state. However, the loss of syt7 abolishes activity-dependent TD (Fig. 6E), which decreases the availability of docked vesicles during subsequent (i.e., two or more) action potentials, indirectly reducing Doc2α-triggered AR during ongoing activity. As a test of this model, an epistasis experiment revealed that the Doc2α/syt7 DKO AR phenotype was non-additive (Fig. 8). This validates a sequential, two-step, model, in which—during ongoing activity—syt7 feeds docked vesicles to Doc2α, which directly mediates AR. Hence, syt7 acts upstream of Doc2α, as a docking factor, while Doc2α functions as the proximal Ca²⁺ sensor for AR in hippocampal synapses. This model can account for why only Doc2α drives AR in response to a single action potential, while Doc2α and syt7 both contribute to AR during subsequent action potentials.

Parameter name	Description	Value	Value determined by
N_{sites}	Number of available release sites (limits the number of docked/tethered vesicles)	200	b (only determines the scaling of the responses)
k_{tet}	Tethering rate	7 s^{-1}	a
k_{untet}	Untethering rate constant	7 s^{-1}	b (same as k_{tet})
k_{docking}	Docking rate constant	5 s^{-1}	a
$k_{\text{undocking}}$	Undocking rate constant	5 s^{-1}	b (same as k_{docking})
I_+	Syt1 and Doc2 independent fusion rate constant / basal fusion rate	$3.5\text{e-}4 \text{ s}^{-1}$	c, Kochubey and Schneggenburger, 2011
Parameters Ca^{2+}-transient			
Amplitude	Amplitude of synchronous Ca^{2+} transient in response to AP	$30 \text{ }\mu\text{M}$	a
Residual Ca^{2+} amplitude	Amplitude of the residual Ca^{2+} signal (following single-exponential decay)	$0.75 \text{ }\mu\text{M}$	a
Tau	Decay time constant of residual Ca^{2+} signal	0.2s	a
$\text{Ca}^{2+}_{\text{rest}}$	Resting calcium concentration	$0.05 \text{ }\mu\text{M}$	c, Helmchen et al., 1997
Parameters syt7			
N_7	Ca^{2+} cooperativity of syt7	2	b, same as for Doc2
K_{D7}	Dissociation constant, syt7	$1.5 \text{ }\mu\text{M}$	c, Brandt et al., 2012
k_{7+}	Ca^{2+} binding rate, syt7	$28 \text{ }\mu\text{M}^{-1} \text{ s}^{-1}$	b, same as for doc2
k_{7-}	Ca^{2+} unbinding rate constant, syt7	42 s^{-1}	c, computed using kd
b_7	Cooperativity factor, syt7	0.5	b, same as for doc2 and syt1
f_7	Effect single Ca^{2+} binding to syt7 has on k_{docking} and $k_{\text{undocking}}$	10 (1 for syt7- simulations)	a
Parameters doc2α			
N_2	Ca^{2+} cooperativity of Doc2 α	2	c, Sun et al., 2007
K_{D2}	Dissociation constant, Doc2 α	$1.5 \text{ }\mu\text{M}$	b (same as for syt7)
k_{2+}	Ca^{2+} binding rate, Doc2 α	$28 \text{ }\mu\text{M}^{-1} \text{ s}^{-1}$	c, (5x reduction compared to syt1, based on Yao et al., 2011; 0 for Doc2 α - simulations.
k_{2-}	Ca^{2+} unbinding rate constant, Doc2 α	42 s^{-1}	c, computed using kd
b_2	Cooperativity factor, Doc2 α	0.5	b, same as for syt1 and syt7
f_2	Effect single Ca^{2+} binding to Doc2 α has on the fusion rate	16	a
Parameters syt1			
N_1	Ca^{2+} cooperativity of Syt1	5	c, Kochubey and Schneggenburger, 2011
k_{1+}	Ca^{2+} binding rate, Syt1	$140 \text{ }\mu\text{M}^{-1} \text{ s}^{-1}$	c, Kochubey and Schneggenburger, 2011
k_{1-}	Ca^{2+} unbinding rate constant, syt1	4000 s^{-1}	c, Kochubey and Schneggenburger, 2011
b_1	Cooperativity factor, syt1	0.5	c, Kochubey and Schneggenburger, 2011
f_1	Effect single Ca^{2+} binding to syt1 has on the fusion rate	27.98	c, Kochubey and Schneggenburger, 2011

Supplementary table 3

a – adjusted to match experimental data, b – set based on additional model assumptions or simplifications, c -based on literature

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Author contributions

Z.W., G.F.K., E.R.C., M.M.M.B., A.M.W., and S.W. designed the study and wrote the manuscript. Z.W. performed all iGluSnFR and electrophysiology experiments, analyzed the corresponding data, and generated the corresponding figures. G.F.K. performed all electron microscopy experiments, with help from S.R., analyzed the corresponding data with S.W., and generated the corresponding figures with S.W. M.M.M.B. and A.M.W. performed simulations. K.I., S.R., and G.F.K. performed knockout cell culture for the electron microscopy experiments. E.R.C., A.M.W. and S.W. funded and oversaw the research.

Ethics declarations

Competing interests

The authors declare no competing interests.

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Article and author information

Zhenyong Wu

Department of Neuroscience, University of Wisconsin-Madison, Madison, USA, Howard Hughes Medical Institute, Madison, USA

Grant F. Kusick

Department of Cell Biology, Johns Hopkins University, School of Medicine, Baltimore, MD, USA, Biochemistry, Cellular and Molecular Biology Graduate Program, Johns Hopkins University, School of Medicine, Baltimore, MD, USA
ORCID iD: [0000-0002-4312-3495](https://orcid.org/0000-0002-4312-3495)

Manon M. M. Berns

Department of Neuroscience, University of Copenhagen, Copenhagen, Denmark

Sumana Raychaudhuri

Department of Cell Biology, Johns Hopkins University, School of Medicine, Baltimore, MD, USA

Kie Itoh

Department of Cell Biology, Johns Hopkins University, School of Medicine, Baltimore, MD, USA

Alexander M. Walter

Department of Neuroscience, University of Copenhagen, Copenhagen, Denmark, Molecular and Theoretical Neuroscience, Leibniz-Institut für Molekulare Pharmakologie, FMP im CharitéCrossOver, Berlin, Germany

ORCID iD: [0000-0001-5646-4750](https://orcid.org/0000-0001-5646-4750)

Edwin R. Chapman

Department of Neuroscience, University of Wisconsin-Madison, Madison, USA, Howard Hughes Medical Institute, Madison, USA

For correspondence: chapman@wisc.edu

Shigeki Watanabe

Department of Cell Biology, Johns Hopkins University, School of Medicine, Baltimore, MD, USA, Solomon H. Snyder Department of Neuroscience, Johns Hopkins University, School of Medicine, Baltimore, MD, USA

For correspondence: shigeki.watanabe@jhmi.edu

ORCID iD: [0000-0001-7580-8141](https://orcid.org/0000-0001-7580-8141)

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Editors

Reviewing Editor

Hugo Bellen

Baylor College of Medicine, Houston, United States of America

Senior Editor

John Huguenard

Stanford University School of Medicine, Stanford, United States of America

Reviewer #1 (Public Review):

Summary:

In the current manuscript, the authors find distinct roles for the calcium sensors Syt7 and Doc2alpha in the regulation of asynchronous release and calcium-dependent synaptic vesicle docking in hippocampal neurons. The authors data indicate that Doc2 functions in activating a component of asynchronous release beginning with the initial stimulus, while Syt7 does not appear to have a role at this early stage. A role for Syt7 in supporting both synchronous and asynchronous release appears during stimulation trains, where Syt7 is proposed to promote synaptic vesicle docking or capture during stimulation. Doc2 mutants show facilitation initially during a train and display higher levels of synchronous release initially, before reaching a similar plateau to controls later in the train. The authors contribute the increased synchronous release in Doc2 mutants to Syt1 having access to more SVs that can fuse

synchronously. In contrast, Syt7 mutants show depression during a train, and continue to decline during stimulation. The authors contribute this to a role for Syt7 in promoting calcium-dependent SV docking and capture that feeds SVs to both synchronous and asynchronous fusion pathways. Importantly, phenotypes of a double Doc2/Syt7 mutant collapse onto the Doc2 phenotype, suggesting the two proteins are not additive in their role in supporting distinct aspects of SV release. Rapid freeze EM after stimulation provides support for a role for Syt7 in SV docking/capture at release sites, as they display less docked SVs after stimulation. In the case of Doc2, EM reveals fewer SVs fusion pits later during a stimulation, consistent with fewer asynchronous fusion events. The authors also provide modeling that supports aspects of their conclusions from the experimental data. I cannot evaluate the modeling data or the specific experimental subtleties of the GluSnFR quantification approach, as these are outside of my reviewer expertise.

Strengths:

The use of multiple approaches (optical imaging, physiology, rapid freeze EM, modeling, double mutant analysis) provides compelling support for distinct roles of the two proteins in regulating SV release.

Weaknesses:

Some of the phenotypes for both Doc2 and Syt7 mutants have been reported in the authors' prior publications. It is not clear how well the GluSnFR approach is for accurately separating synchronous versus asynchronous release kinetics. The authors also tend to overstate the significance of the two proteins for asynchronous release in general, as a significant fraction of this release component is still intact in the double mutant, indicating these two proteins are only part of the asynchronous release mechanism.

<https://doi.org/10.7554/eLife.90632.2.sa1>

Reviewer #2 (Public Review):

Summary:

The goal of this study is to provide a deeper understanding of the roles of syt7 and Doc2 in synaptic vesicle fusion. Depending on the system studied, and the nature of the preparation, it appears that syt7 functions as a sensor for asynchronous release, synaptic facilitation, both processes, or neither. The perspective offered by Chapman, Watanabe, and colleagues varies from those previously published, and is therefore novel and interesting.

Strengths:

The strengths of the study include the complementary imaging and electrophysiology approaches for assessing the function of syt7, and the use of appropriate knockout lines. High resolution imaging approaches to measure synaptic activity is also a strength.

Weaknesses:

It is not clear to this reviewer that the computational modeling effort is important or even necessary. The study also attempts to derive kinetic information (on the ms time scale) from EM. While the interpretations are not unreasonable, they should be taken with some caution.

Overall, the study does a good job of attempting to resolve the various ambiguities existing in the field regarding the potential roles of syt7 and Doc2 in membrane fusion. There are, of course, a great number of proteins which have been identified to act at fusion sites to drive or otherwise modify release phenotypes. Efforts such as this are going to become increasingly important as we work to attribute discrete roles to each one.

Author Response

The following is the authors' response to the original reviews.

First, we discovered several erroneous duplicate values in our source data sets from figures S1, 2, 4, and 8, due to mistakes from MATLAB analysis. We have re-analyzed the data and corrected these errors; since limited values in each data set changed, the results were unaffected. The changes are reflected in updated figures and source data.

Overall, the reviewers gave a positive assessment of our work, but had reservations about:

- (1) Specifics of the iGluSnFR data and analysis
- (2) Overstatement/oversimplification of the importance of syt7 and Doc2
- (3) The strength and interpretation of the EM data
- (4) The relevance and parametrization of the modeling data

(1) We have clarified aspects of the iGluSnFR data and analysis in the point-by-point response, as well as in the manuscript.

(2) We have toned down our statements about the role of syt7 and Doc2 throughout, and emphasized that the DKO data are conclusive and reveal that there must be additional Ca^{2+} sensors for AR. We have also added to the discussion, noting syt3 as a strong candidate to perform a function analogous to syt7 (to regulate docking), along with another protein (or proteins) performing a role similar to Doc2 (directly in fusion) that has not been identified as a candidate in the field yet.

(3) We feel the EM data are consistent with the model as much as they could be, and while a sequence of events can only be inferred from time-resolved EM, we believe our work falls in the scope of reasonable interpretation. However, upon reexamining the terminology of 'feeding' and related discussion, we realized this could be misleading, so these sections have been revised.

(4) We have improved the description and interpretation of the model in the manuscript and provide a detailed rationale of our approach in the point-by-point-response.

Reviewer #1 (Recommendations For The Authors):

Major points:

(1) It is surprising the optical GluSnFR approach reports so much asynchronous release in control hippocampal neurons after single stimuli (36% of release). This seems much higher than what is observed at most synapses, where asynchronous release is usually less than 5% of the initial response to the first evoked stimuli. Any thoughts on why the GluSnFR approach reports such a high level of asynchronous release? Could the optical approach be slower in activation kinetics in some cases, which artificially elevates the asynchronous aspect of fusion? This seems to be the case, given electrophysiology recordings in Figure 3 show the asynchronous release component as ~10% in controls at the 1st stimuli (panel C).

The reported proportion of asynchronous release from cultured hippocampal neurons varies, contingent upon a range of factors (calcium concentration, how asynchronous release is quantified, etc). However, we would argue that there is considerable evidence for a higher percentage of asynchronous release (more than the <5% indicated by the referee) at synapses

in the hippocampus. In our previous work on Doc2 using electrophysiology in cultured hippocampal neurons (Yao et al., 2011, Cell), it was noted that there is an approximate 25% incidence of asynchronous release after a single action potential. Furthermore, Hagler and Goda also reported a 26% ratio of asynchronous neurotransmitter release, also from cultured hippocampal neurons (Hagler and Goda, 2001, J Neurophysiol.).

We also point out that another study using iGluSnFR to measure synchronous/asynchronous release ratios, with more sophisticated stimulation, imaging, and analysis procedures than ours, found an average ratio of synchronous to asynchronous release that is in-line with our values, with considerable variability among individual boutons (Mendonça et al., 2022; 25% asynchronous release after a single action potential). We feel that iGluSnFR is actually the superior approach (barring specialized e-phys preparations that can measure quantal events at individual small synapses; please see Miki et al., 2018), as it directly measures the timing of individual release events at individual boutons. By comparison, in most electrophysiology experiments there is a large peak of synchronous release from many synapses. iGluSnFR also bypasses postsynaptic considerations such as receptor kinetics and desensitization, or asynchronous release being poorly aligned to AMPA receptors, per a recent study of ours (Li et al., 2021), and a study showing 25% of asynchronous release occurs outside the active zone (Malagon et al., 2023). All these factors could obscure asynchronous release or otherwise make it difficult to measure by electrophysiology. To our knowledge, the approach in Miki et al., 2018 best bypasses these limitations, though the data in that study are from exceptionally fast and synchronous cerebellar synapses, and so cannot be directly compared to our findings. Thus, it is possible that iGluSnFR can report more asynchronous release than electrophysiological recordings, but this may actually reflect real biology.

This being said, after considering the reviewer's points we realized that our analysis method likely underestimates the total amount of synchronous release when using the high-affinity sensor (Figure 1). We quantify release by 'events' (that is, peaks), which does not take into account multiquantal peaks resulting from near-simultaneous multivesicular release. We have previously determined by quantal analysis that most synchronous peaks after a single action potential are multiquantal, while for asynchronous release there are still multiquantal events but they are in the minority (Vevea et al., 2021; Mendonça et al., 2022). So, in our data sets, the total amount of synchronous release is underestimated more so than asynchronous release. Thus, 37% asynchronous release is probably an overestimate, which explains the 12% difference compared to Mendonça et al., 2022, who used sophisticated quantal analysis (though that study also was performed at room temperature, which could also cause differences). We have now pointed this out in the text:

"This ratio of synchronous to asynchronous release is likely an underestimate, since our analysis only counts the number of peaks ('events') and does not take into account multiquantal peaks resulting from near-simultaneous multivesicular release. We have previously determined by quantal analysis that most synchronous peaks are multiquantal after a single action potential, while for AR there are still multiquantal events but they are in the minority (Vevea et al., 2021). So, in our measurements, the total amount of synchronous release is underestimated; sophisticated quantal analysis using the A184V iGluSnFR recently found the percentage of total release that is AR to be ~25%, with otherwise similar results to ours (Mendonça et al., 2022). Nonetheless, this approach faithfully distinguishes synchronous from asynchronous release..."

However, while this method underestimates total synchronous release, it does not misclassify synchronous events as asynchronous because of kinetics. Even the slower iGluSnFR variant does not have a rise time that would misrepresent a synchronous event as asynchronous (Marvin et al., 2018). Mendonça et al (2022) note that averaged iGluSnFR traces for the A184V are biphasic, with the transition from fast to slow component occurring around 10 ms. These authors also determined that the temporal resolution of glutamate imaging is actually limited

by the frame rate, not the biosensor, and based on simulations found that detection time was biased in their data to be about 1 ms earlier than the actual timing of release events.

The reviewer's final point about Figure 3 is a misunderstanding, as these are data from iGluSnFR, not electrophysiology. The asynchronous proportion in these experiments is ~10% because, as noted in the manuscript, we used a faster, lower-affinity variant of iGluSnFR in train stimulation experiments (Figure 2). In contrast to the high-affinity sensor, as explained above, in our analysis this variant would be expected to underestimate the amount of asynchronous release because it fails to detect many unquantal release events (presumably those further from the focal plane, with too little fluorescence to reach our detection threshold) as evidenced by the fact that the apparent mini rate is much lower as measured by this sensor compared to higher-affinity variants. Since synchronous peaks are mostly multiquantal after a single action potential, while asynchronous peaks are mostly unquantal, a fraction of release going undetected results in mostly smaller synchronous peaks, which are counted the same in our analysis while many asynchronous peaks are missed entirely. We have added a bit more clarification in the text to avoid confusion on this point:

"This sensor underestimates the fraction of AR (~10% of total release for a single action potential) as compared to the A184V variant used above that overestimates the fraction of AR (~35% of total release for a single action potential). This is because it is less sensitive and misses many unquantal events; as discussed above, our analysis quantifies release by number of peaks, and most synchronous peaks are multiquantal after a single action potential, while most AR peaks are unquantal (Vevea et al., 2021). Still, the S72A variant reported the same phenotypes as the A184V variant after the first action potential (Fig. 3B, C)."

As discussed above, we think the synchronous-to-asynchronous ratio is actually harder to determine with electrophysiology, and the preparations are different (acute slice vs dissociated culture); still, our electrophysiological measurements are in line with the iGluSnFR data: 29% for Figure 2 and 26% from the first action potential of Figure 4. These values also agree with the findings from Yao et al. (2011) and Hagler and Goda (2001), discussed above.

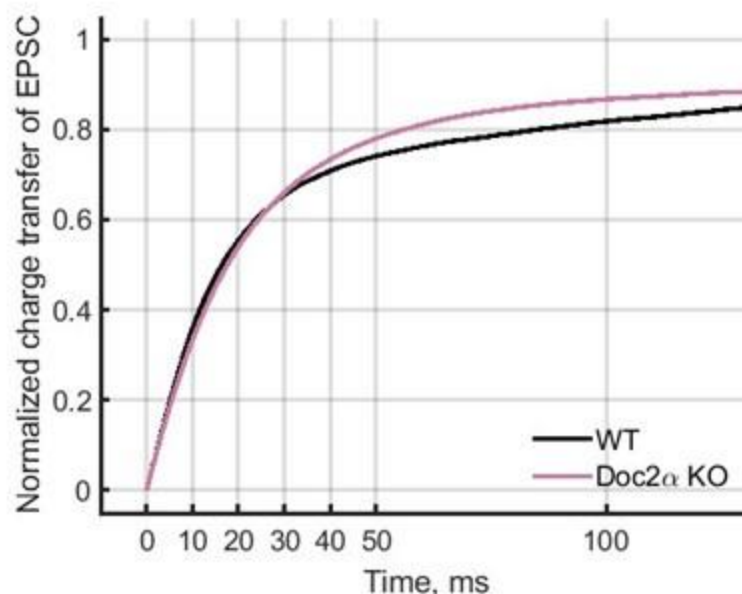
Finally, the ultimate goal of our study was to measure the effects of deleting Doc2 and syt7 on synchronous and asynchronous release, not to measure the exact ratio between the two. If iGluSnFR greatly misreported synchronous events as asynchronous, we would expect the results from the knockouts to diverge between our imaging and electrophysiology data, which they do not. We have also previously applied this approach to syt1 knockouts, showing the characteristic desynchronization of release (Vevea et al., 2020). Furthermore, the high-affinity and low-affinity iGluSnFR variants, which as discussed above in our analysis overestimate and underestimate the fraction of release that is asynchronous, respectively, both reported the same phenotypes.

(2) In the acute hippocampal physiology traces, it looks like the effect on cumulative release in Doc2A mutants only appears around ~40 msec after stimulation. This is a relatively late phase of asynchronous release. Any reason this effect does not show up sooner, where most asynchronous fusion events occur, or is this due to some technical aspects of the physiology clamp that masks earlier components?

The reviewer is correct, although the curves actually diverge at around 30 ms (see image below). This can be attributed to the fact that the EPSCs in our recordings are broad, probably because of the large number of different synaptic inputs captured in our stimulation and recording paradigm (note that the currents are also quite large), resulting in a broad spread in the timing of release. That is to say, synchronous release is likely still occurring fairly late into the trace, obscuring any changes in asynchronous release earlier than 30 ms. This is not

related to Doc2 specifically, as the EGTA charge transfer curve also diverges from the control curve at the same time. This EGTA control gives us confidence that our broad EPSCs still faithfully report synchronous and asynchronous release, even if the exact timing is spread-out to some extent.

Author response image 1.



(3) How do the authors treat multi-vesicular release in their synchronous/asynchronous quantification? It was not clear from the methods section. Many of the optical traces show dual peaks - are those that occur in the 10 ms bin assigned to synchronous and those outside to asynchronous? Are the authors measuring the area of the response or just the peak amplitude for the measurements? The methods seem to indicate peak amplitude, but asynchronous is better quantified with area measurements for electrophysiology.

This is an excellent point by the reviewer, and in the Methods we now explicitly state how we treat multivesicular release/multiple peaks in our analysis. Release timing is assigned based on peak timing, including when there are multiple peaks at the same bouton.

“Timing of release was determined based on the frame in which the signal peaked, including for dual peaks in the case of synchronous and asynchronous release at the same bouton.”

Regarding the comparison to area measurements for electrophysiology, we agree with the reviewer, which is why we used such an approach for our electrophysiological data. However, a key advantage of iGluSnFR is the ability to resolve individual quantal events (or, as is often the case for synchronous release, simultaneous multiquantal events), so temporal binning of the peaks is the appropriate analysis approach regarding these data. This is comparable to the analysis used for electrophysiology recordings of responses from single small synapses, which also detects individual quantal events, where release timing is calculated as the latency between the stimulus and the beginning of each EPSC (Miki et al., 2018).

This leaves the general concern that multiple vesicle fusions at the same bouton that occur milliseconds apart could blur together and make it more difficult to accurately determine

release timing, particularly with the slower sensor used in the single-stim experiments in Figure 1. We believe this is not a major concern, since we also performed experiments with the much faster sensor, S72A which can resolve peaks from 100 Hz stimulation (Marvin et al., 2018). Furthermore, while the peak-calling method we used is crude by comparison, the synchronous/asynchronous ratio we report is similar to that of Mendonça et al. (2022) who used a higher frame rate and deconvolution to produce more easily distinguishable quanta when synchronous and asynchronous release occur at the same bouton after the same action potential.

(4) It would be relevant to show that calcium binding mutations in Syt7 do not support SV docking/capture in the current assays, given some evidence for Syt7 calcium-independent activities has been reported in the field.

To our knowledge, when using the correct mutations to block calcium binding, none of the reported syt7 knockout phenotypes (including those reported by our laboratory in Liu et al., 2014) have ever been rescued. However, this does not formally rule out a calcium-independent role in transient docking. For the EM data, we originally considered including rescue experiments with normal and non-calcium binding mutants of both syt7 and Doc2 in our study. However, our EM approach is spectacularly expensive and labor-intensive and such experiments would as much as triple the amount of EM work in the study. We plan on doing such experiments, and there is a great deal of additional structure-function work to be done on both these proteins. We feel that reassessing the calcium binding mutants with iGluSnFR and zap-and-freeze falls into the scope of this future work. For now, this as a limitation of the current study.

(5) The authors are not consistent in how they describe the role of the two proteins in asynchronous release, with the reader often drawing the impression that these two proteins solely mediate this aspect of SV fusion. As the authors note, some synapses do not require Syt7 or Doc2 for SV release, indicating different asynchronous sensors or molecular components at distinct brain synapses. Indeed, asynchronous release is only reduced, not eliminated, in the double mutants the authors report, so other components are at play even in these hippocampal synapses. The authors should be more consistent in noting this in their text, as the wording can be confusing as noted below:

"Together, these data further indicated that AR after single action potentials is driven by Doc2a, but not syt7, in excitatory mouse hippocampal synapses."

"after a single action potential, Doc2a accounts for 54-67% of AR at hippocampal excitatory synapses, whereas deleting syt7 has no effect."

"This, along with our finding that syt7/Doc2a DKO still had remaining AR, raises the possibility that there are other unidentified calcium sensors for AR."

We have made adjustments throughout to not overstate the role of syt7 and Doc2, including at the locations the reviewer points out. This is an important point from the reviewer, and not just to avoid misleading readers. It is itself interesting; in the original manuscript we should have emphasized, far more than we did, that the DKO experiments strongly point to as yet unidentified proteins being involved in asynchronous release. This has been rectified in the revised text: we now emphasize that another calcium sensor for asynchronous release is likely present at all relevant points in the manuscript.

(6) Given the authors' data, I don't think it's fair to say "raises the possibility" of other AR sensors, as almost 50% of AR remained in the Doc2A mutant in some of the experimental approaches. Clearly, other AR calcium sensors or molecular components are required, so better to just state that in the 1st paragraph of the discussion with something like: "Given

*syt7/Doc2a DKO*s still had remaining AR, further work should explore the diversity of synaptic Ca^{2+} sensors and how they contribute to heterogeneity in synaptic transmission throughout the brain."

We agree; this was poor phrasing on our part. We meant to imply that there *may* be proteins that have not even been considered, because it is also technically possible that the remaining asynchronous release is supported by the known machinery (i.e., syt1). We have changed "raises the possibility" to "indicates".

Minor points:

(1) Remove "on" from the abstract sentence "Consequently, both synchronous and asynchronous release depress from the second pulse on during repetitive activity".

We have changed "on" to "onward" to reduce ambiguity.

(2) Shouldn't syt7 be Syt7 and syt1 be Syt1 when referring to the proteins?

To our knowledge there is not a hard-and-fast convention for non-acronym mouse protein abbreviations. The technically correct full name is lowercase, so we find it reasonable to use lowercase for the abbreviation.

(3) Both calcium and Ca^{2+} are used in the manuscript - better to stick to one term throughout.

We thank the referee for catching this error; we now use only " Ca^{2+} " throughout our study.

Reviewer #2 (Recommendations For The Authors):

(1) While the iGluSnFR experiments appear to be well done, what is striking is the relatively small and "jagged" fluorescent responses. Are the authors concerned that they are missing many fast (with peaks occurring within 10 ms) synchronous events and incorrectly identifying them asynchronous? If this is not a concern, why not?

With respect to the small raw responses, this is the nature of measuring individual quanta from individual boutons while imaging at 100 Hz, even with the excellent signal-to-noise ratio of the iGluSnFR variants we used.

As far as kinetics, as noted in the response to Reviewer 1 point #1, even the slower iGluSnFR variant has a rise time fast enough that it cannot misrepresent a synchronous event as asynchronous (Marvin et al., 2018). This threshold for iGluSnFR has been used by others: see Mendonça et al., 2022, who note that averaged iGluSnFR traces are biphasic, with the transition from fast to slow component occurring around 10 ms. The 'jaggedness' is in large part due to the frame rate (100 Hz); Mendonça et al., 2022 used 250 Hz and deconvolution to produce smoother, cleaner traces, but still achieved similar results to us.

Finally, we reiterate what we wrote in response to Reviewer 1 point #1: "the ultimate goal of our study was to measure the effects of deleting Doc2 and syt7 on synchronous and asynchronous release, not to measure the exact ratio between the two. If iGluSnFR misreported synchronous events as asynchronous, we would expect the results from the knockouts to diverge between those data and our electrophysiology data, which they do not. We have also previously applied this approach to syt1 knockouts, showing the characteristic desynchronization of release (Vevea et al., 2020). Also, the phenotypes reported by the faster and slower iGluSnFR variants were identical."

(2) On page 6, I'm not sure I would agree that short-term plasticity is "so catastrophically disrupted". It is probably enough to say that plasticity is disrupted in the ko.

We argue that syt7 knockout causes the most severe phenotype specific to short-term plasticity so far described (that is, without affecting initial release probability), but we have changed “catastrophically” to “strongly”.

(3) Differences in the post-stim number of "docked" vesicles between conditions are, in absolute numbers, very small. For example, it seems that the number of docked vesicles goes from ~ 2.2 prior to stimulation, to ~ 1.5 in the first 5 ms window following stimulation. While this number may be statistically significant, I worry about bias and sampling errors. It is comforting that images are randomized prior to analysis. Nevertheless, the differences are very small and this should be explicitly acknowledged.

This ~40% decrease in number of docked vesicles in dissociated cultured hippocampal neurons has been consistent throughout all our studies using flash-and-freeze and zap-and-freeze electron microscopy (Watanabe et al., 2013; Kusick et al., 2020, Li et al., 2021), as well as those of other labs (Chang et al., 2018). Statistically, 40% is far beyond the limit to detect differences between samples with 200-300 synapses quantified per condition and an average of ~2 docked vesicles per image. The low absolute number of docked vesicles per synaptic profile (since the 40 nm section only captures a portion of the active zone, which contain an average of 12 docked vesicles in total; Kusick et al., 2020) is not relevant except that it does reduce the statistical power to detect differences, but this is compensated for by the huge number of images we capture and annotate per sample. We are able to detect differences in fusion and endocytic pits (albeit with much less precision and sensitivity), such as the Doc2 phenotype in this study, even though these events are an order of magnitude rarer than docked vesicles. Biologically, in our view, a 40% reduction in all docked vesicles across all synapses, considering that the majority of synapses do not have even 1 vesicle fusion, after only a single action potential, is substantial. We have even been puzzled why there is such a large decrease, but as stated above this result has been consistent for a decade of using this approach. For comparison to the magnitude of baseline docking changes in mutants, this 40% is similar to the effect of deleting synaptotagmin 1 (Imig et al, 2014; Chang et al, 2018; note in Imig et al., considered a gold standard in the field, the average number of docked vesicles per tomogram is ~10, but there are fewer than 25 tomograms per sample, so the actual amount of sampling in our data set is slightly greater).

(4) The related point is that how can one know about the "transient" nature of vesicle docking when the analysis is performed on completely different sections from different cells? Moreover, what does it mean that the docked granules have recovered or not recovered (abstract)? This should be explained in more detail.

This is a fundamental difficulty of interpreting time-resolved electron microscopy data. We cannot observe a sequence of events at any given synapse, but only try to measure each time point as accurately as we can and interpret the data.

By ‘recovery’ we simply mean that the number of docked vesicles at a given time point after stimulation is similar to the no-stimulation baseline. We have replaced ‘recovery’ in the abstract with ‘replenishment’ to avoid confusion.

We now realize that in the context of this study the term ‘transient docking’ is confusing, since we only measured out to 14 ms in this study. In experiments with samples frozen at 5 ms, 14 ms, 100 ms, 1 s and 10 s, the return to baseline at 14 ms appears temporary, since samples frozen at 100 ms have a similar reduction of docked vesicles as those at 5 ms (Kusick et al., 2020). The number of vesicles again returns to baseline at 10 s, so we used the term

‘transient docking’ to distinguish the recovery at 14 ms from the slower and presumably permanent return to baseline that takes 10 s. The apparently temporary nature of this process is why we believe it contributes to facilitation, which likewise peaks soon after stimulation and decays over the course of ~100 ms.

To make the transient docking terminology less confusing, we have removed the word ‘transiently’ from the title and added a clarification of what transient docking is when it is first mentioned:

“vesicles can dock within 15 ms of an action potential to replenish vacated release sites and undock over the next 100 ms”

As noted by the reviewer, such a sequence of events, where vesicles dock within 14 ms, then undock over the course of 100 ms, then dock again over the course of 10 s, is an inference, but is based on predictions from electrophysiological data and modeling (see Silva, Tran, and Marty, 2021 for review; those authors use the term ‘calcium-dependent docking’ but this refers to the same process), and as yet there is no way to directly observe vesicle dynamics at synapses down to nanometer resolution in live cells.

On the reviewers recommendation we have removed references to syt7 ‘feeding’ vesicles from the abstract and the beginning of the “physiological relevance” section of the discussion. This phrasing could imply a direct molecular pipeline between syt7 and syt1/Doc2, which is a misrepresentation of our actual model that syt7 simply helps recruit docked vesicles.

“These findings result in a new model whereby syt7 drives activity-dependent docking, thus providing synaptic vesicles for synchronous (syt1) and asynchronous (Doc2 and other unidentified sensors) release during ongoing transmission.”

“In the case of paired-pulse facilitation it can supply docked vesicles for syt1-mediated synchronous release to enhance signaling; it likely functions in the same manner to reduce synaptic depression during train stimulation. In the case of AR, syt-7-mediated docked vesicles can be used by Doc2α, which then directly triggers this slow mode of transmission.”

(5) In this study, docking is phenomenologically defined and, therefore, arbitrary; vesicles are defined as docked if there is no space between them and the plasma membrane. What happens if the definition is broadened to include some small distance between the respective membranes? Does the timecourse of "recovery" change?

We always quantify at least all vesicles within 100 nm of the active zone; these data are shown in Figure S6D. We show only docking in the main figures because, consistent with our previous work and as stated in the text, we found no change in the number of vesicles at any distance from the plasma membrane at the active zone after stimulation, nor did we find any difference in the mutants. In our previous work on syt7 (Vevea et al., 2021) we quantified all the vesicles within the synapse and also found no differences after stimulation or in the KO further from the active zone.

The reviewer is correct that the term ‘docking’ at synapses is often used quite arbitrarily; even among morphological studies the definition is inconsistent. We consider our strict docking definition that we explain in the manuscript (in high-pressure-frozen and freeze-substituted samples) of no visible distance between membranes to be less arbitrary, since only the number of these attached vesicles decreases after stimulation (Watanabe et al., 2013, Kusick et al., 2020, Li et al., 2021, this study) and in SNARE knockouts (Imig et al., 2014). Broadening the definition, as is done in some other studies (for example Chang et al., 2018), retains the effect, since the majority of vesicles within 10 nm are at ~0 nm, but again all that is actually changing is the number of vesicles at ~0 nm.

(6) My overall impression is that this model is not adding much to the story. Specifically, the model was not fit to any data and has a huge number of states and free parameters given the dynamics that it is trying to capture (ie I think this is overkill). Many of the free parameters were arbitrarily constrained with little to no justification and there was minimal parameter space exploration, in part because the model wasn't being quantitatively constrained to any data. While advertised to be a 3-state model, there is a combinatorial explosion of substates by distinguishing between levels of calcium occupancy simultaneously in three separate calcium sensors so that one ends up with 9 empty states, 9 tethered states, and 45 docked states for a total of 63 distinguishable states. At 63 states and 21 free parameters, one could of course model just about any dynamics imaginable. But the relatively simple dynamics of AR and its perturbation by removal of Doc2 and Syt7 can likely be captured with far fewer states and parameters (such as Neher's recent proposal). Specifically, starting with the Neher ES-LS-TS model along with adding a transient labile docked state affected by Syt7 and Doc2 (TSL in Neher nomenclature), I wonder if the authors could more or less capture what they are observing during stimulus trains. The advantage of a minimal model is that readers don't have to struggle with fairly elaborate systems of differential equations and parameter plots to get a feel for what's going on. Especially since the point of this model is to develop intuition rather than to capture with physical accuracy exactly what is transpiring at a docked vesicle (which would require many more details excluded from the current model).

We would like to thank the reviewer for pointing out unclarities and mistakes in the description of the model. We have worked on improving on these points. We now more elaborately explain why we have made certain assumptions and what decisions we have made to constrain the parameter values in the model. As the reviewer points out other models might also work in explaining the dynamics of the experimental data presented in this paper. Thus, we agree that it is unlikely that this theory and model implementation is the only one that can account for the observations. With this model we aimed to investigate whether the theory proposed based on the experimental data could indeed reproduce the dynamics that are observed experimentally. In the section below we will briefly explain why we made different decisions in constructing the model to comment on the reviewer's concerns. We will also discuss more precisely what adjustments we have made to the model's description to improve its readability and be open about its limitations.

One of the main concerns of the reviewer is that the model has many states and free parameters, some of which are poorly constrained. We agree that the model indeed contains many states. However, in essence, the model corresponds to a two-step docking model, in which SVs get tethered to an empty release site and subsequently dock/prime in a fusion-competent state. This structure of the model corresponds to the ES-LS-TS model (Neher and Brose 2018, Neuron) mentioned by the reviewer or the replacement-docking model (Miki et al., 2016, Neuron). As the reviewer points out, by making the transition rates calcium-dependent in those models, we would indeed be able to capture similar dynamics with these models as with ours. However, instead of directly implementing calcium-dependent rates, we let the rates depend on the number of calcium ions bound to syt7, Doc2 and Syt1. We decided to do so, as some information on the calcium binding dynamics of these proteins is available. By simulating the calcium binding to the proteins explicitly we could integrate this knowledge into our model. Moreover, by explicitly simulating calcium-binding to these proteins, we included the time it takes before a new steady state-binding occupancy is reached after a change of calcium levels. Especially for Ca^{2+} sensors with slow kinetics such as, syt7 and Doc2, this is crucial. These properties are highly relevant for asynchronous release (which we quantified as the release >5 ms after onset of AP). The consequence is that because of combinatorics (e.g., if we assume 5 calcium ions to bind to syt1 and 2 to Doc2 this

leads to 24 different states), explicit simulation of all relevant states extends the number of potential different states a vesicle can be in. In the main text of the manuscript, we added this explanation on why we decided on the structure of the model as it is presented and discussed it in context of other previous models.

Our decision to simulate calcium binding to syt1, syt7 and Doc2 also increased the number of parameters in our model. As the reviewer points out, the large number of parameters in our model compared to the relative low number of features in the experimental behavior the model is compared to – is a limitation. However, after thorough exploration of the model, we are certain that the model cannot create any type of desired dynamics. The large number of parameters does make it possible that different combinations of parameter values would lead to similar responses, as can be seen in the parameter space exploration in Figure S9. This means that our modelling effort does not provide estimates of parameter values. We now mention this explicitly in the discussion section of the model. Some of the parameter values we were able to constrain based on previous literature (10 parameters), others were more arbitrary set (8 parameters), and some of them were adjusted to match the experimental data closely (7 parameters). We indicated more clearly now in Supplementary Table 3 to which category each parameter value belongs in table. We determined the values of the model parameters through a manual exploration of the parameter space. One of the main reasons why we decided not to perform a fitting of the model to data obtained in this work is that the obtained parameters would not be informative (e.g., multiple combinations of parameters will lead to similar results). We agree with the reviewer that a direct quantitative comparison between model predictions and experimental data obtained by fitting would be nice. However, fitting the model to experimental data would be close to impossible computationally. This is in part because of the large number of states, but mainly due to the large number of APs that need to be simulated. Especially since the transients in our model have slow and fast parts (the decay of the residual Ca^{2+} -transient, and the peak of the local Ca^{2+} -transient), the model is challenging to solve with ODE solvers available in Matlab, even when using a high-performance computer system optimized for parallel computation (32 cores). Moreover, fitting the model to experimental data would require the addition of extra assumptions and parameters to the model. As the experiments are performed using different samples, different parameter settings are probably required (e.g. it is likely that the number of release site or the fusion probability differs between cultured hippocampal neurons and hippocampal slices). Additionally, if we decide to fit the model, we would need to define a cost function (i.e., a quantitative measure of how well the model is fitting to experimental data), which requires us to determine the different weights the different experiments we are comparing our model predictions to have. The decision on how to weight the different types of data is very difficult (not to say arbitrary).

Therefore, we constrained the parameter values in our model based on a manual (but systematic) exploration of the parameter space. The simulations of the model were evaluated based on the increase in the number of docked vesicles between 5 and 15 ms after AP stimulation (this should be as large as possible for the control and Doc2- model, and close to 0 for the syt7- model simulations), the peak release rates in response to the first AP (to be equal between all conditions), the ratio between the peak release rate of the 1st and 10th response (depressive phenotype should be more prominent in the syt7- model simulation and the least in the Doc2- simulation), and the amount of asynchronous release (syt7- and Doc2- simulations should have approximately half of the total amount of asynchronously released vesicles compared to the control simulations). Moreover, the parameter values for the calcium transient should be realistic. We do not know the exact parameter values of the calcium transient in the samples used in the experiments performed here, but previous studies have provided a range of realistic parameter values (Brenowitz and Regehr 2007, PMID: 17652580; Helmchen et al., 1998, PMID: 9138591; Sabatini and Regehr 1998, PMID: 9512051; Wang et al., 2008, PMID: 19118179). Furthermore, we decided to set the parameters describing calcium binding to syt7 and Doc2 to the same values, as the scope of the model

was to investigate the role of syt7 and Doc2 in asynchronous release when they act on different steps in the reaction scheme. By using the same parameter values both proteins are identical except for their mechanism of action. We added this section to the methods of the manuscript.

In the parameter space evaluation, we decided to vary parameters one-by-one or in pairs of two. We decided not to further extend the parameter space evaluation as it will be challenging to give a proper interpretation of these results, to visualize them, and to simulate it (computationally expensive).

(7) The graphics, equations, and nomenclature all need some work. The equations aren't numbered or indexed, so I can't really refer to any of them in particular, but the symbols being used generally were not defined well enough for a naïve reader to follow. The 15 diffEQs compressed into a single expression at the bottom of page 19 are basically impenetrable. The 'equation' near the bottom of p. 20 is not an equation - it is a set of four symbols lacking a definition. The fusion rate equation (with f1 and f2 factors) isn't spelled out clearly enough (top of p. 20). Can fusion occur from any of the 45 docked states but just with a different probability? Or does fusion only occur from the 3 states where Doc2+Syt1 Ca occupancy = 5? The graphical representation of Syt7 occupancy and its effects in Fig S7 doesn't work well. Tons of color and detail but very hard to decipher and intuit what Syt7 is doing to the SV buried in the arrow lengths. And this is a crucial point of the paper - it really needs to shine through in this figure.

We thank the reviewer for pointing out the unclarities in the description of the model. We have worked on improving this section. Specifically, we have improved the equations and now more clearly explain the symbols used in these equations. We have altered the graphical representation of the effect of calcium binding to syt7 on docking and undocking rates.

(8) I would strongly recommend abandoning this large-scale soft modeling effort altogether, but if the authors feel that all the states and parameters are absolutely required, they need to justify this point, define all symbols systematically, number all equations, and provide some evidence of actual data fitting, systematic parameter space exploration, and more exposition of why they are making the various assumptions and constraints that were used to lower the number of free parameters. For instance, why are the tethering and untethering (or docking and undocking) rate constants set to equal each other? And why is it assumed that Syt7 enhances both the docking and undocking rates? Why is fusion set to occur as long as the sum of Syt1 and Doc2 calcium occupancy is exactly 5 regardless of the specific occupancy of either Syt1 or Doc2? Again probably quite important but unjustified physically. Given the efforts of this model to capture some sort of realistic calcium liganding by Syt1, Syt7, and Doc2, the model doesn't seem to take into account the copy number of each protein at a release site. Shouldn't it matter if there are 2 Syt7s vs 20 Syt7s? Or the stoichiometry between Doc2 and Syt1? Either this model assumes that there is exactly one copy of each protein at a release site or that all copies are always identically liganded and strictly act as a unit. Neither of these possibilities seems plausible.

Despite the fact that this model (as all models) is a simplified version of reality and despite the fact that this model (as all models) has its limitations, we decided to keep the model in our work to illustrate that this well-defined hypothesis put forth in this paper is consistent with the experimental data. Again, we are not claiming that this model is the only one that may explain this, nor do we claim that we have uniquely identified its parameters. As indicated above, we worked on improving the description of the model in the methods and improved on our description of how the parameter values are constrained. For the reasons mentioned above (first and foremost because of infeasibility due to excessive computation time) we did

not perform data fitting or changed the parameter space exploration. We would like to thank the reviewer for pointing out that some of the assumptions of the model are not well enough explained. We added an extra explanation of these assumptions to the main text.

One of the assumptions we made, as the reviewer points out, is that the tethering and untethering and docking and undocking rates constants are set to equal each other. This is indeed an arbitrary assumption, with the main aim of reducing the number of free parameters in our model given that there is currently no experimental constraint on the relation between the two rate constants. We agree that this assumption is as good as any other, and we have pointed this out more clearly in the main text.

In the model syt7 enhances both docking and undocking rates as we assumed it to function as a catalyst of the docking reaction. A catalyst lowers the energy barrier for the reaction and thereby promotes both forward and backward rates. One of the main reasons we decided on this is because in the model also syt1 and Doc2 are assumed to function by lowering the energy barrier for the fusion reaction. However, since fusion is irreversible this would only affect the forward reaction rate. We cannot exclude that syt7 acts on the forward rate only, which we now mention in the results section of the model.

In our model fusion can occur from any possible docked SV state. The probability of fusion however increases the more calcium ions are bound to Doc2 or Syt1, with Syt1-bound to Calcium being more effective in promoting fusion. This structure matches the dual-sensor model proposed by Sun et al., 2007, Science (PMID: 18046404) and Kobbersmed et al. 2020, Elife (PMID: 32077852), and is based on the assumption that each protein bound to calcium lowers the energy barrier with a certain amount. We have explained this more in the results section of the model.

We decided that syt1 and Doc2 together could have no more than five calcium ions bound to them. This is based on the idea that syt1 and Doc2 are competing for the same type of resources, which could for instance be a limited number of SNARE complexes that are available to execute the reaction. An indication for competition between the two proteins can be found in the synchronous release amplitudes after stimulus 2, which are larger in the Doc2KO.

The reviewer rightfully points out that for realistic simulations of the role of syt1, syt7 and Doc2 the stoichiometry of these proteins at the release site is relevant. In the ideal scenario, we would have included this in our model. However, this would massively increase the possible number of states (which this reviewer criticizes already in our simpler model), making the model even more computationally expensive to run. Additionally, we currently have no reliable estimates of the number of syt7 and Doc2 molecules per release site. In our model, all syt1s expressed on an SV can bind up to five calcium ions. We have recently shown that this simplified model can capture the features of all syt1 proteins per vesicle that compete for the binding of three substrates on the plasma membrane to exert their function in speeding up fusion (Kobbersmed et al., 2022 eLife PMID: 35929728). This means that the copy number is indirectly covered in our model. This number of five calcium ions (and two for Doc2 and syt7) however is not based on the estimated number of syt1s on an SV (which would be around 15, Takamori 2006), but rather on the calcium-dependence of the fusion reaction. Similarly, the number of two calcium ions binding to Doc2 is based on the Calcium-dependence of asynchronous fusion rates (Sun et al., 2007). Based on the reviewer's comment we now more explicitly mention in the text that the numbers of calcium ions binding to syt1, Doc2 and syt7 corresponds to the total number of calcium ions that can bind to each of these molecules per release site/SV.

We again would like to thank the reviewer for asking us to improve the explanation on the assumptions made to construct our model and how we constrained the parameter values in our model.