The calcium sensor synaptotagmin 7 is required for synaptic facilitation

Skyler L. Jackman1, Josef Turecek1, Justine E. Belinsky1 & Wade G. Regehr1

It has been known for more than 70 years that synaptic strength is dynamically regulated in a use-dependent manner. At synapses with a low initial release probability, closely spaced presynaptic action potentials can result in facilitation, a short-term form of enhancement in which each subsequent action potential evokes greater neurotransmitter release. Facilitation can enhance neurotransmitter release considerably and can profoundly influence information transfer across synapses, but the underlying mechanism remains a mystery. One proposed mechanism is that a specialized calcium sensor for facilitation transiently increases the probability of release, and this sensor is distinct from the fast sensors that mediate rapid neurotransmitter release. Yet such a sensor has never been identified, and its very existence has been disputed. Here we show that synaptotagmin 7 (Syt7) is a calcium sensor that is required for facilitation at several central synapses. In Syt7-knockout mice, facilitation is eliminated even though the initial probability of release and the presynaptic residual calcium signals are unaltered. Expression of wild-type Syt7 in presynaptic neurons restored facilitation, whereas expression of a mutated Syt7 with a calcium-insensitive C2A domain did not. By revealing the role of Syt7 in synaptic facilitation, these results resolve a longstanding debate about a widespread form of short-term plasticity, and will enable future studies that may lead to a deeper understanding of the functional importance of facilitation.

Several mechanisms for facilitation have been proposed (Extended Data Fig. 1). In the ‘buffer saturation’ model, high concentrations of presynaptic calcium buffer capture incoming calcium before it binds to the rapid synaptotagmin isoforms (1, 2 and 9) that trigger vesicle fusion at most synapses. If the calcium buffer saturates during the first action potential, more calcium reaches release sites during subsequent action potentials, producing facilitation. Yet many facilitating synapses lack sufficient presynaptic calcium buffer to account for this form of facilitation. Another theory suggests that a specialized calcium sensor responds to the smaller, longer-lasting calcium signals that trigger action potentials. In one scenario, this sensor modulates calcium channels to produce use-dependent increases in calcium influx. Several candidate proteins have been proposed to act in this manner, but increased calcium influx cannot account for facilitation at most synapses. Alternatively, an unidentified calcium sensor could mediate facilitation by directly increasing the probability of release.

Syt7 is located presynaptically, and binds calcium with high affinity and slow kinetics, making it a promising candidate sensor for the modest increases in residual calcium that mediate facilitation. Previous studies suggest that Syt7 contributes to a slow phase of neurotransmitter release known as asynchronous release, and to calcium-dependent recovery from depression, but the role of Syt7 in facilitation was not examined because these studies used synapses with prominent depression that obscures facilitation. We therefore examined synaptic transmission at four facilitating synapses: Schaffer collateral synapses between hippocampal CA3 and CA1 pyramidal cells (Fig. 1a), thalamocortical synapses between layer 6 cortical pyramidal cells and thalamic relay cells (Fig. 1b), mossy fibre synapses between dentate granule and CA3 cells (Fig. 1c), and perforant path synapses between layer II and III cells of the entorhinal cortex and dentate granule cells (Fig. 1d). Immunohistochemistry shows that Syt7 is present in regions where these synapses are located (Extended Data Figs 2 and 3). Facilitation is often assessed using pairs of closely spaced stimuli. In slices from wild-type mice, paired-pulse facilitation resulted in ~2-fold enhancement of neurotransmitter release lasting several hundred milliseconds (Fig. 1a–d, black traces). In Syt7-knockout mice, paired-pulse facilitation was eliminated (Fig. 1a–d, red traces). Sustained high frequency activation produces up to 10-fold enhancement in wild-type animals, but facilitation is eliminated in knockouts at all synapses except for mossy fibre synapses, where the remaining enhancement is consistent with use-dependent spike broadening that occurs at this synapse (Fig. 1e–h and Extended Data Fig. 4).

The loss of facilitation in Syt7 knockout cannot be accounted for by slowed recovery from depression reported with Syt7 deletion, because recovery from depression is too slow to influence rapid facilitation strongly, nor can it produce the large increase in release associated with facilitation. There are several possible explanations for the loss of facilitation in knockouts: (1) the presynaptic calcium signal that induces facilitation could be altered, (2) the probability of release (p) for synaptic vesicles could be increased, which by promoting vesicle depletion would indirectly reduce facilitation, or (3) the mechanism for facilitation could be disrupted directly. We assessed these possibilities at the CA3–CA1 synapse.

Action-potential-evoked increases in presynaptic calcium consist of a large, brief localized calcium signal that activates the low-affinity calcium sensor synaptotagmin 1 to trigger neurotransmitter release, and a small residual calcium signal (Ca_res) that persists for tens of milliseconds and has been implicated in facilitation. It is difficult to measure local calcium signals that trigger release, but Ca_res is readily measured. We used a low-affinity calcium indicator to measure the time course of Ca_res in CA3 presynaptic terminals, because facilitation can be attenuated by the accelerated decay of Ca_res (ref. 4). Ca_res decayed similarly in wild-type and Syt7-knockout animals (Fig. 2a), indicating that the loss of facilitation in knockout mice is not a consequence of accelerated Ca_res decay. We also used Ca_res as a measure of Ca_influx to determine whether there are use-dependent changes in calcium entry. However, each of two closely spaced stimuli evoked the same incremental increase in Ca_res in both wild types and knockouts (Fig. 2b), indicating that use-dependent changes in total Ca_influx cannot account for facilitation. This suggests that if changes in Ca_influx contribute to facilitation at this synapse, they must be restricted to the small subset of presynaptic calcium channels that evoke neurotransmitter release. We repeated the experiment using a high-affinity calcium indicator, in which the degree of saturation during paired stimuli can be used to measure the magnitude of Ca_influx evoked by the first stimulus (see Methods). We conclude that Ca_influx evoked by the first stimulus is the same in wild-type and knockout animals (Fig. 2c).

We further explored the role of calcium in facilitation by examining the calcium-dependence of excitatory postsynaptic currents (EPSCs)
Figure 1 | Facilitation is absent in Syt7 knockout mice.

a–d, Representative traces (top) and average paired-pulse ratio (PPR) at different interstimulus intervals ($\Delta t$) (bottom) recorded in slices prepared from wild-type (WT; black) and Syt7-knockout (KO; red) animals. Postsynaptic responses were recorded using whole-cell voltage clamp from hippocampal CA1 pyramidal cells (a), and thalamic relay cells (b). fEPSPs were recorded from hippocampal-mossy-fibre to CA3 synapses (c), and lateral-perforant-path synapses in the dentate gyrus (d).

and facilitation. Raising extracellular Ca$^{2+}$ leads to a steep increase in EPSC amplitude (Fig. 2d) but a decrease in facilitation (Fig. 2e, black traces), even though high extracellular Ca$^{2+}$ should increase the $Ca_{res}$ available to evoke facilitation. This paradox is resolved by realizing that increased Ca$^{2+}$ influx increases $p$, which depletes presynaptic vesicles, saturates release and limits the extent of facilitation. The Ca$^{2+}$-dependence of EPSC amplitudes was unaffected in knockout animals (Fig. 2d), but facilitation was absent for all values of external Ca$^{2+}$ (Fig. 2e, f). Meanwhile, there was no difference in basal release properties measured by the rate of spontaneous EPSCs (Extended Data Fig. 5). These findings suggest that the loss of facilitation in knockouts is not a consequence of higher initial $p$, because facilitation was absent even when the initial $p$ was strongly attenuated by reducing external Ca$^{2+}$.

To test further whether initial $p$ is increased in Syt7 knockouts, we measured how field excitatory postsynaptic potentials (fEPSPs) scaled with stimulus intensity (Fig. 3a). The slope of the fEPSP versus presynaptic volley gives a relative measure of $p$ (see Methods), which was unchanged in knockouts (Fig. 3b). Moreover, the fEPSP to presynaptic volley ratio changed steeply with extracellular Ca$^{2+}$, showing that this method is sensitive to $p$ (Fig. 3c, d). We also assessed $p$ using pharmacological blockade of synaptically activated NMDARs (N-methyl-D-aspartate receptors) by the use-dependent blocker MK801 (ref. 25; Fig. 3e–g). This approach is widely used to detect changes in $p$: an increase in $p$ leads to more glutamate release, and more activation and rapid blockade of NMDARs, while a decrease in $p$ leads to a slower blockade (Extended Data Fig. 6). The rate of blockade of NMDAR-mediated fEPSPs (NMDAR-fEPSPs) was unaffected by Syt7 deletion (Fig. 3e), indicating similar initial $p$. However, when we evoked NMDAR-fEPSPs with trains of three stimuli, amplitudes decayed more rapidly in wild types (Fig. 3f, g), suggesting that Syt7 is required to increase $p$ for the second and third stimuli. Thus, initial $p$ and presynaptic Ca$^{2+}$ signalling are unaffected by Syt7 deletion, but knockouts lack the use-dependent increase in $p$ that underlies facilitation. This suggests that the mechanism underlying facilitation is directly impaired by Syt7 deletion.

Syt7 is implicated in neuroendocrine release16, insulin secretion26 and exocytosis of lysosomes27, which could all indirectly influence synaptic transmission in global Syt7 knockouts. Therefore, to determine whether Syt7 controls facilitation by acting in presynaptic neurons in a cell-autonomous manner, we tested whether viral expression of Syt7 in CA3 pyramidal cells of Syt7 knockouts rescued facilitation. This approach is complicated by our inability to virally transduce all CA3 pyramidal cells, which prohibits the use of extracellular stimulation that would activate some presynaptic cells that express Syt7 and others that do not. We overcame this problem with an adeno-associated virus (AAV) that drove bicistronic expression of both channelrhodopsin-2 (ChR2) and Syt7, allowing optical stimulation of only those fibres expressing Syt7.

Using conditions we have previously shown allow facilitation to be studied with optogenetic stimulation (see Methods), we confirmed that when ChR2 alone was expressed, optical and electrical stimulation produced similar facilitation in wild types (Fig. 4a, e, f), and similar depression in knockouts (Fig. 4b, e, f). We next used a bicistronic vector to express both ChR2 and wild-type Syt7 in knockout animals. Light-evoked responses exhibited facilitation, whereas electrically evoked responses did not (Fig. 4c, e, f). This suggests that bicistronic expression of ChR2 along with a presynaptic protein of interest offers a powerful new approach to characterize the effect of gene manipulation on presynaptic function within intact neural circuits. When Syt7 was expressed in wild-type animals, the peak facilitation was unaffected (Fig. 4e, f and Extended Data Fig. 7a). Thus, expressing Syt7 in CA3 pyramidal cells rescued facilitation in a cell-autonomous manner, with facilitation restored only at synapses expressing Syt7 and ChR2.
Figure 2 | Facilitation is altered in Syt7-knockout animals despite similar presynaptic Ca\(^{2+}\) signals. a, Presynaptic Ca\(_{\text{res}}\) evoked by a single stimulus recorded from Schaffer collateral fibres loaded with a low-affinity Ca\(^{2+}\) indicator (left), and Ca\(_{\text{res}}\) half-decay times (right). NS, not significant. b, Ca\(_{\text{res}}\) signals recorded with low-affinity indicator evoked by one or two stimuli (left). The ratio of the increase in Ca\(_{\text{res}}\) evoked by the first (\(\Delta F_1\)) and second (\(\Delta F_2\)) stimuli (right). c, Ca\(_{\text{res}}\) signals recorded with high-affinity indicator evoked by one or two stimuli. d, Average EPSC amplitudes for CA3–CA1 synapses recorded in different external Ca\(^{2+}\) (Ca\(_{\text{e}}\)) concentrations, normalized to the amplitude in 2 mM Ca\(_{\text{e}}\). e, EPSCs recorded in different Ca\(_{\text{e}}\). Vertical scale bars, 50, 100, 200 and 300 pA in 0.5, 1, 2 and 3 mM Ca\(_{\text{e}}\), respectively. f, PPR for interstimulus interval of 20 ms recorded in different Ca\(_{\text{e}}\). In 0.5 mM Ca\(^{2+}\), the PPR in knockout (1.24 ± 0.12) was not significantly different from 1 (\(P = 0.084\), Wilcoxon signed rank test). Data represent mean ± s.e.m. Number of experiments is shown in Extended Data Table 2.

Figure 3 | Change in the initial probability of release does not underlie the absence of facilitation in Syt7-knockout mice. a, Extracellular recordings of presynaptic fibre volley and fEPSP evoked by the indicated stimulus intensities. Scale bar, 200 μV. b, fEPSP slope plotted against fibre volley amplitude, for 20–100 μA stimulation. c, fEPSPs recorded in 1 and 3 mM Ca\(_{\text{e}}\). Scale bar, 100 μV. d, Average ratio of the fEPSP to the fibre volley in different Ca\(_{\text{e}}\). e, Top, initial release probability was measured by stimulating Schaffer collaterals every 10 s while recording NMDAR-fEPSPs before and after MK801 bath application. Middle, traces averaged from 10 trials before (dark traces), and trials 10–15 after (light traces) MK801 application. Bottom, average NMDAR-fEPSP amplitudes evoked in the presence of MK801. f, Same as e but with three stimuli at 50 Hz every 30 s. First response to trains is shown. g, Half-decay times of NMDAR-fEPSP amplitudes in the presence of MK801. * \(P < 0.05\), one-way analysis of variance (ANOVA) with Tukey’s post-hoc test. Data represent mean ± s.e.m. Number of experiments shown in Extended Data Table 2.

Figure 4 | Viral expression of Syt7 restores facilitation at Schaffer collateral synapses. a–d, Top, fluorescence images of yellow fluorescent protein (YFP)-tagged ChR2 and Syt7 immunostaining in the CA1 region after AAV injection into CA3 to express the indicated proteins in wild-type animals (a) or Syt7-KO animals (b–d). PY, stratum pyramidale; SR, stratum radiatum. Scale bar, 100 μm. Bottom, EPSCs and PPRs for responses evoked electrically (open symbols) and optically (blue symbols). In a and b, only ChR2–YFP was expressed; in c, both ChR2–YFP and wild-type Syt7 were expressed (separated by a porcine teschovirus-1 2A (P2A) cleavage peptide); and in d, ChR2–YFP and Ca\(^{2+}\)-insensitive Syt7(C2A\(^{*}\)) were expressed. e, f, Summary of PPRs for 50-ms interstimulus interval. Asterisks denote significant difference from responses evoked electrically in un.injected wild-type animals (e), or optically in wild-type animals expressing ChR2 alone (f). * \(P < 0.05\), one-way ANOVA with Tukey’s post-hoc test. Data represent mean ± s.e.m. Number of experiments is shown on bar graphs.

To determine whether Ca\(^{2+}\) binding by Syt7 is important for facilitation, we assessed whether facilitation is rescued by Syt7 with a mutated Ca\(^{2+}\)-insensitive C2A domain (Syt7(C2A\(^{*}\))). Previous studies established that Ca\(^{2+}\) binding to the C2A domain of Syt7 is required for Syt7 to mediate asynchronous release. We found that Syt7(C2A\(^{*}\)) did not rescue facilitation in knockout (Fig. 4d–f). Moreover, in wild-type animals, Syt7(C2A\(^{*}\)) expression strongly attenuated facilitation (Fig. 4e, f and Extended Data Fig. 7b), suggesting that Syt7(C2A\(^{*}\)) competes with native Syt7 to suppress facilitation.

Our results indicate that facilitation requires Ca\(^{2+}\) binding to the C2A domain of Syt7, and also provide insight into the role of Syt7 in facilitation. We conclude that Syt7 does not produce facilitation by altering the amplitude and time course of Ca\(_{\text{res}}\) (Fig. 2), by increasing initial \(p\) (Fig. 3), by acting as a Ca\(^{2+}\)-insensitive C2A domain (Syt7(C2A\(^{*}\))). Previous studies established that Ca\(^{2+}\) binding to the C2A domain of Syt7 is required for Syt7 to mediate asynchronous release. We found that Syt7(C2A\(^{*}\)) did not rescue facilitation in knockout (Fig. 4d–f). Moreover, in wild-type animals, Syt7(C2A\(^{*}\)) expression strongly attenuated facilitation (Fig. 4e, f and Extended Data Fig. 7b), suggesting that Syt7(C2A\(^{*}\)) competes with native Syt7 to suppress facilitation.
Syt7 remains an open question. It is also unclear whether the recently described interaction between Syt7 and calmodulin that promotes vesicle replenishment is similarly required for facilitation. Finally, it is possible that at other synapses facilitation is mediated by additional specialized Ca\(^{2+}\) sensors, or involves other mechanisms. Further studies are needed to clarify these issues.

Based primarily on theoretical considerations, facilitation is thought to influence both information transfer and network dynamics profoundly. In the hippocampus, the high-pass filtering imposed by facilitating synapses may account for the burst firing in place cells that encode spatial information \(^{28}\). In the auditory pathway, facilitation is proposed to counteract short-term depression to maintain linear transmission of rate-coded sound intensity \(^ {29} \). It has even been suggested that facilitation forms the basis of short-term memory, as facilitated transmission of rate-coded sound intensity is known to encode spatial information \(^ {28} \). In the auditory pathway, facilitation is thought to enable the persistent activity states associated with working memory \(^ {30} \). In future studies, the selective elimination of Syt7 from specific cell types could allow the first direct tests of the effect of facilitation on neural circuits and behaviour.

**Online Content** Methods, along with any additional Extended Data display items and any additional references are available in the online version of the paper.

**Author Contributions** S.L.J. performed electrophysiology, and J.T. measured Ca\(^{2+}\) currents at presynaptic nerve terminals. S.L.J. and J.T. measured Ca\(^{2+}\) currents during synaptic facilitation using the rat calyx of Held. J. Physiol. (Lond.) 586, 5503–5520 (2008).

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METHODS

Animals and viruses. All mice were handled in accordance with NIH guidelines and protocols approved by Harvard Medical School. Sy7 knockout mice11 (Jackson Laboratory) and wild-type littermates of either sex were used. Statistical tests were not used to predetermine sample size. Blinding and randomization were not performed. AA2V/9-hsyn-hChR2(H134R)-EYFP and its pAAV backbone (Addgene 26973) were obtained from the University of Pennsylvania Vector Core. Complementary DNA encoding the rat Sy7 wild-type α isoform and C2A* mutant (D253A, D257A and D233A)13 were provided by T. Bacaj and T. Sudhof. For rescue experiments involving Sy7 with mutated Ca2+ binding domains, we used the mutated C2A* version instead of the C2A* C2B* double mutant, as mutation of both C2 domains leads to lower levels of expression. The P2A cleavage sequence12 and Sy7 were inserted after the ChR2 carboxy terminus in the pAAV backbone (GenScript). Plasmid-driven expression of ChR2–YFP and Syt7 was confirmed in HEK cells by Sy7 immunostaining and patch-clamp recording of ChR2 photorecords. AAVs were produced and purified from HEK cells as previously described13.

Stereotoxic surgeries were performed as described13. Postnatal day (P) 18–30 mice were anaesthetized with ketamine/xylazine/neuromazine (100/10/3 mg kg−1) supplemented with 1–4% isoflurane. Viruses were injected through glass capillary needles using a syringe (Hamilton) mounted on a stereotaxic instrument (Kopf). Injection coordinates from lambda were 2.69 mm (rostral), 3 mm (lateral) and 2.8 mm (ventral). One microlitre of virus suspension was delivered at a rate of 0.1 μl min−1 using a microsyringe pump (WPI; UMP3) and microsyringe pump controller (WPI; Micro4). The needle was slowly retracted 5–10 min after injection, and the scalp incision was closed with glue. Post-injection anaesthetic (buprenorphine, 0.05 mg kg−1) was administered subcutaneously for 48 h.

Acute slice preparation. P30–P60 animals were euthanized under isoflurane anaesthesia, 14–30 days after AAV injection. Brains were removed and placed in ice-cold solution containing (in mM): 234 sucrose, 25 NaHCO3, 11 glucose, 7 MgCl2, 2.5 KCl, 1.5 NaH2PO4 and 0.5 CaCl2. Then, 270-μm-thick transverse slices (hippocampal recordings) or 250-μm-thick sagittal slices (thalamic recordings) were prepared on a vibrotome (Leica, VT1000s), and a cut was monitored during recordings. fEPSPs were recorded in current-clamp mode with −70 mV, and series resistance was monitored during recordings. iEPSPs were recorded in current-clamp mode with ACSF-filled patch pipettes (0.5–1 MΩ). Inhibition was blocked with picrotoxin (50 μM), and during iEPSP recordings, CPP (2 μM) and CPG (3 μM) was added to the bath. Approximately 4–10 trials were conducted for each stimulus frequency, and recordings were averaged over trials. Data in all figures represent the mean ± s.e.m. Average responses are displayed with double exponential or polynomial curves fit in IgorPro. Unless stated otherwise, statistical significance was assessed by unpaired two-tailed Student’s t-test, or one-way ANOVA followed by Tukey’s post-hoc test.

Probability of release. To record NMDAR-iEPSPs, cells were voltage clamped at +40 mV, and the internal solution contained (in mM): 85 Cs-methanesulfonate, 4 NaCl, 10 HEPES, 0.2 EGTA, 30 BAPTA, 2 MgATP, 0.4 NaGTP, 10 phospho-crea-tine, Na; 25 TEA, 5 QX-314; pH 7.3. For recording NMDAR-iEPSPs, Mg2+ was excluded from ACSF to relieve Mg2+ block of NMDA receptors. Picrotoxin (100 μM) and NBQX (5 μM) were added to the bath, and stimulation was conducted at 0.1 Hz (unless otherwise indicated) for 5 min to obtain a baseline response. Stimulation was halted for 10 min while (+)-MK801 (40 μM) was added and allowed to equilibrate. For experiments involving iEPSPs versus presynaptic volley, the postsynaptic response was measured by the slope of the iEPSP, while the amplitude of the presynaptic volley was used to determine the number of activated fibres. If increases, the same number of activated presynaptic fibres will produce a larger iEPSP. The ratio between iEPSP and volley was determined by line fits to the linear regime of the input–output curve of individual experiments (20–80 μA stimuli).

The study of probability of release is complicated because many people use p to refer to the probability of release of a vesicle (p_r) and others refer to probability of release from an active zone (p_{synapse}) that contains N vesicles in its readily releasable pool. Thus, an increase in the size of the readily releasable pool for an active zone can increase p_{synapse} even if p_r is unaltered. Although MK801 blockade22 and iEPSPs versus presynaptic volley are22 both widely used methods to detect changes in the probability of release, for both approaches it is conceivable (although unlikely) that increases in p_r could be obscured by a perfectly balanced decrease in the readily releasable pool size. However, the relationship between EPSC amplitude and extracellular Ca2+ is similar in wild-type and Sy7-knockout animals. This suggests there is no increase in p_r, which would cause this curve to saturate at lower values of Ca_{syn} for Sy7-knockout animals. Moreover, the large differences in facilitation in wild-type and Syt7-knockout animals were even more pronounced when the probability of release was reduced tenfold by lowering Ca_{syn} from 2 mM to 0.5 mM, which is incompatible with an increase in p_r obscuring facilitation by depleting vesicles.

Ca2+ measurements. Ca2+ was measured as described previously4. In brief, CA3 fibres were labelled for 3 min using an AAV-filled pipette containing either magnesium green AM or fura-2 AM (240 μM) and 1% fast green, placed into the border of the CA3–CA1 field. A vacuum pipette placed above the loading site removed excess indicator. Slices were incubated for at least 1 h and imaging was performed in stratum radiatum of CA1 at least 500 μm from the injection site using a 60× objective and custom-built photodiode. Excitation was achieved using a tungsten (magnesium green) or xenon (fura-2) lamp. Schaffer collateral stimuli were stimulated using a glass electrode placed at 300 μm from the imaging site. To prevent recurrent excitation, experiments were performed in the presence of NBQX (10 μM), CPP (2 μM) and picrotoxin (50 μM).

Mg2+ and calcium green is a low-affinity calcium indicator46 (K0 = 7 μM) that provides a linear measure of Ca_{syn} (ref. 37). As such it is well suited to measuring the time course of presynaptic Ca_{syn} (Fig. 2a) and detecting changes in Ca_{syn} during successive stimulations (Fig. 2b). However, with the bulk loading approach the size of the fluorescence change is proportional to the number of stimulated fibres, so the absolute Ca_{syn} signal is not readily quantified with magnesium green. By contrast, fura-2 has a high affinity for calcium48,29 (K0 = 131 mM) so it provides a saturating sublinear response to increases in Ca_{syn} (refs 40–42). This can be used to test for changes in the absolute size of Ca_{syn} because a change in the Ca_{syn} per stimulus would change the ratio between the fluorescence change observed in its readily releasable pool. Thus, an increase in the size of the readily releasable pool for an active zone can increase p_{synapse} even if p_r is unaltered. Although MK801 blockade22 and iEPSPs versus presynaptic volley22 are both widely used methods to detect changes in the probability of release, for both approaches it is conceivable (although unlikely) that increases in p_r could be obscured by a perfectly balanced decrease in the readily releasable pool size. However, the relationship between EPSC amplitude and extracellular Ca2+ is similar in wild-type and Syt7-knockout animals. This suggests there is no increase in p_r, which would cause this curve to saturate at lower values of Ca_{syn} for Sy7-knockout animals. Moreover, the large differences in facilitation in wild-type and Syt7-knockout animals were even more pronounced when the probability of release was reduced tenfold by lowering Ca_{syn} from 2 mM to 0.5 mM, which is incompatible with an increase in p_r obscuring facilitation by depleting vesicles. Ca2+ measurements. Ca2+ was measured as described previously4. In brief, CA3 fibres were labelled for 3 min using an AAV-filled pipette containing either magnesium green AM or fura-2 AM (240 μM) and 1% fast green, placed into the border of the CA3–CA1 field. A vacuum pipette placed above the loading site removed excess indicator. Slices were incubated for at least 1 h and imaging was performed in stratum radiatum of CA1 at least 500 μm from the injection site using a 60× objective and custom-built photodiode. Excitation was achieved using a tungsten (magnesium green) or xenon (fura-2) lamp. Schaffer collateral stimuli were stimulated using a glass electrode placed at 300 μm from the imaging site. To prevent recurrent excitation, experiments were performed in the presence of NBQX (10 μM), CPP (2 μM) and picrotoxin (50 μM).
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Extended Data Figure 1 | Possible mechanisms for synaptic facilitation. a–d, It is established that calcium has an important role in synaptic facilitation, and several mechanisms have been proposed that involve different aspects of calcium signalling. Here we discuss the calcium signals that evoke rapid vesicle fusion, and also those thought to be involved in facilitation (a), and three mechanisms of facilitation are presented schematically (b–d). a, To understand the mechanisms that have been proposed to account for facilitation, it is important to appreciate different aspects of presynaptic calcium signalling. Calcium signals are complex, but can be approximated by two components. An action potential opens calcium channels for less than a millisecond, and near open channels the calcium levels reach tens of micromolar. Release sites near calcium channels experience high local calcium levels (Ca\textsubscript{local}) that are highly dependent on the distance from open calcium channels. Ca\textsubscript{local} can be reduced by high concentrations of fast calcium buffers that rapidly bind calcium. In addition, there is a residual calcium signal (Ca\textsubscript{res}) that results from calcium equilibrating within presynaptic terminals, before calcium is gradually removed over tens of hundreds of milliseconds. The amplitude of Ca\textsubscript{res} (and also total influx of Ca\textsuperscript{2+}, Ca\textsubscript{influx}) is determined by all of the calcium channels that open, not only those that produce Ca\textsubscript{local} that drives release, and after initial equilibration Ca\textsubscript{res} is roughly uniform throughout the presynaptic bouton. It is generally accepted that fast synaptic transmission is produced by calcium binding to Syt1, Syt2 or Syt9, which have low-affinity binding sites, fast kinetics, and require the binding of multiple calcium ions. The time course of release follows the time course of calcium channel opening, but with a brief delay (<1 ms). Ca\textsubscript{res} after a single stimulus is much smaller than Ca\textsubscript{local}. Typical fluorescence-based approaches to measure calcium readily detect Ca\textsubscript{res}, but are insensitive to Ca\textsubscript{local}, which is too localized and short-lived to measure. Note the y axis is logarithmic to show both Ca\textsubscript{local} and Ca\textsubscript{res} in a, but not in b–d. b, For one mechanism of facilitation, a fast calcium buffer is present in presynaptic terminals that binds calcium and reduces Ca\textsubscript{local}. Stimulation twice in rapid succession results in the same calcium influx for both stimuli. If there is no fast presynaptic buffer, the amplitudes of Ca\textsubscript{local} and the EPSCs are the same for both stimuli (red traces). If a fast high-affinity buffer is present (black traces), it reduces the initial Ca\textsubscript{local} and reduces the amplitude of the initial EPSC, but if enough calcium enters and binds to the buffer, it reduces its ability to buffer calcium. As a result, the second stimulus produces larger Ca\textsubscript{local} than the first, and the EPSC is facilitated. c, A second possible mechanism is that more calcium enters for the second stimulus, and as a result there is more neurotransmitter release. This could arise from a spike broadening, or from the modulation of calcium channels. It is possible that influx through all calcium channels in the presynaptic terminal would be increased, in which case both Ca\textsubscript{local} and Ca\textsubscript{res} would be increased. It is also possible that the only calcium channels that are modulated are the subset that produce Ca\textsubscript{local} that triggers release, in which case Ca\textsubscript{res} would not be significantly increased. d, Finally, it is possible that there is a specialized calcium sensor that produces facilitation that is distinct from Syt1 (refs 2, 4, 45). Previous studies have shown that such a sensor would need to be sensitive to Ca\textsubscript{res} based on the observation that facilitation is altered at some synapses by manipulations that affect Ca\textsubscript{res} without affecting Ca\textsubscript{local}. According to this scheme, release is mediated by Syt1 but calcium binding to a second sensor would increase p. The sensor is sufficiently slow that it does not influence release evoked by the first stimulus, but it is able to influence release evoked by a second stimulus.
Extended Data Figure 2 | Immunohistochemistry of Syt7 expression at four different synapses. 
a–d, Fluorescent images of immunostaining for vGlut1 (top) and syt7 (bottom) in slices from wild-type and Syt7-knockout animals, showing the stratum radiatum (SR) of hippocampal CA1 region (a), the ventral thalamus (b), mossy fibres (MF) in hippocampal CA3 (c), and the lateral and medial perforant paths (LPP and MPP) in the outer molecular layer of the dentate gyrus (d). Notably, Syt7 expression in wild-type animals was higher in the LPP, where synapses exhibit facilitation, than in the MPP, where synapses exhibit depression. Scale bar, 50 μm.

The presence of Syt7 labelling in regions containing CA3–CA1 synapses, layer 5 to thalamus synapses, mossy fibres synapses and LPP–granule-cell synapses that are also colabelled with antibodies to the presynaptic marker for glutamatergic synapses vGlut1, suggests that Syt7 is located presynaptically at these synapses. It is, however, difficult to obtain sufficient resolution with confocal microscopy in brain slices to unambiguously establish that Syt7 is located presynaptically at these synapses. Importantly, the Allen Brain atlas (http://www.brain-map.org) suggests that the presynaptic cells for these synapses contain messenger RNA for Syt7. Lastly, immunoelectron microscopy revealed selective staining of presynaptic boutons in the CA1 region of the hippocampus.
Extended Data Figure 3 | Immunohistochemistry of Syt7 and calbindin expression at mossy fibre synapses. Fluorescent images of immunostaining for calbindin-D28k, which predominantly labels mossy fibres in the CA3 region of the hippocampus⁹⁻¹⁶ (top) and Syt7 (bottom) in slices from wild-type and Syt7-knockout animals. Colocalization of Syt7 and calbindin staining in wild-type animals provides further support for the expression of Syt7 in mossy fibre terminals. Scale bar, 20 μm.
Extended Data Figure 4 | Loss of facilitation in Syt7-knockout animals at multiple frequencies. Average normalized synaptic responses evoked by extracellular stimulation with trains at frequencies from 5 to 50 Hz at four synapses in slices from wild-type and Syt7-knockout animals. Enhancement during trains was eliminated for all synapses other than mossy fibre synapses, where significant enhancement was present by the fifth stimulus for 5 Hz and 10 Hz, the third stimulus for 20 Hz, and the sixth stimulus for 50 Hz (compared to 1 by a Wilcoxon signed rank test, \( P < 0.05 \)). This indicates that another form of synaptic enhancement gradually builds during repetitive activation and is consistent with a specialized form of synaptic enhancement that has been described at mossy fibre synapses in which spike broadening gradually builds during repetitive activation and leads to increased calcium influx. The numbers of experiments are shown in Extended Data Table 1.
Extended Data Figure 5 | Spontaneous release is similar in wild-type and Syt7-knockout animals. a, Representative spontaneous EPSCs (sEPSCs) recorded from voltage-clamped hippocampal CA1 cells in wild-type (black) and knockout (red) animals. Vertical scale bars, 20 pA. 

b, Representative sEPSCs, averaged from >50 events recorded in wild-type and knockout animals. Vertical scale bars, 10 pA. 

c, d, Average sEPSC amplitude (c) and frequency (d) in wild-type (n = 16) and Syt7-knockout animals (n = 18).
Extended Data Figure 6 | MK801 blockade of NMDAR-mediated EPSCs reveals similar initial release probability in wild-type and knockout synapses. a, Representative NMDAR-EPSCs recorded in wild-type and knockout animals before the application of MK801 (average of 10 traces) and after stimulation in the presence of MK801 (average response of fifteenth to twentieth stimuli). Vertical scale bars, 100 pA. b, Average NMDAR-EPSCs recorded in the presence of MK801, normalized to the first stimulus. c, Half-decay times of NMDAR-EPSC amplitudes. *P < 0.05, one-way ANOVA with Tukey’s post-hoc test. Data represent mean ± s.e.m. The number of experiments is shown in Extended Data Table 2.
Extended Data Figure 7 | Effect of virally expressed Syt7 wild-type and Syt7(C2A*) in wild-type animals. a, b, Top, AAV was injected into the hippocampal CA3 region in wild-type animals to express ChR2 and either wild-type Syt7 (a) or Syt7(C2A*). Bottom, representative EPSCs and average paired-pulse ratios for responses evoked electrically and optically in wild-type slices with AAV-driven expression of wild-type Syt7 (electrical, \( n = 12 \); optical, \( n = 13 \)) (a) and Syt7(C2A*) (electrical, \( n = 5 \); optical, \( n = 13 \)) (b). Vertical scale bars, 100 pA.
Evidence suggests that Syt7 does not produce facilitation by acting as a local calcium buffer at the CA3–CA1 synapse. This graph illustrates the general relationship between PPR and external calcium for synapses in which buffer saturation produces facilitation (green) and for facilitation observed at the CA3–CA1 synapse and many other synapses (black). It has been shown previously that the for buffer saturation mechanism (Extended Data Fig. 1b) the amplitude of facilitation is reduced when Ca$_{influx}$ is reduced by lowering external calcium. This can be understood by considering that this form of facilitation is thought to require sufficient Ca$_{influx}$ to saturate the endogenous buffer, and thereby reduce its ability to buffer calcium for subsequent stimuli. If Ca$_{influx}$ is low, then there is insufficient calcium entry to bind very much of the endogenous buffer, and little facilitation would result. In addition, as shown in Extended Data Fig. 1, for a calcium buffer to produce facilitation it would need to buffer calcium sufficiently that it would reduce initial $p$. We have shown, however, that $p$ is unaltered in Syt7 knockouts. This is perhaps not surprising in light of the fact that Syt7 is thought to be located on the plasma membrane, and in cases where this type of facilitation has been observed it is associated with high concentrations of a fast cytosolic buffer.
Extended Data Table 1  |  Number of electrophysiological recordings from wild-type and Syt7-knockout animals

| Figure   | Synapse                  | Experiment   | Genotype | # of Recordings | # of Animals |
|----------|--------------------------|--------------|----------|-----------------|--------------|
| Figure 1a| Schaffer collateral      | Paired-pulse | WT       | 13              | 6            |
| Figure 1b| Corticothalamic          | Paired-pulse | KO       | 17              | 6            |
| Figure 1c| Hippocampal mossy fibre | Paired-pulse | WT       | 23              | 8            |
| Figure 1d| Lateral perforant path   | Paired-pulse | KO       | 23              | 5            |
| Figure 1e| Schaffer collateral      | Trains (2-50 Hz) | WT | 10              | 6            |
| Data Figure 4| Corticothalamic | Trains (2-50 Hz) | KO | 8               | 4            |
| Figure 1f| Corticothalamic          | Trains (2 Hz) | WT   | 16              | 8            |
| Data Figure 4| Corticothalamic | Trains (5 Hz) | KO   | 5               | 2            |
| Figure 1g| Hippocampal mossy fibre | Train (2 Hz) | WT | 12              | 3            |
| Data Figure 4| Corticothalamic | Trains (5 Hz) | KO | 3               | 2            |
| Figure 1h| Lateral perforant path   | Train (2 Hz) | WT   | 10              | 5            |
| Data Figure 4| Lateral perforant path | Train (2-50 Hz) | KO | 3               | 2            |
| Figure 1i| Lateral perforant path   | Train (10 Hz) | WT | 5               | 2            |
| Data Figure 4| Lateral perforant path | Train (20 Hz) | KO | 3               | 3            |
### Extended Data Table 2 | Number of experiments related to the Ca\(^{2+}\)-dependence of probability of release

| Figure | Experiment | Condition | Genotype | # of Recordings | # of Animals |
|--------|------------|-----------|----------|-----------------|--------------|
| Figure 2a,b | Presynaptic | Magnesium Green | WT | 11 | 2 |
| Ca\(^{2+}\) Imaging | KO | 10 | 2 |
| Figure 2c | Presynaptic | Fura-2 | WT | 14 | 3 |
| Ca\(^{2+}\) imaging | KO | 10 | 2 |
| Figure 2d-f | Ca\(^{2+}\)-dependence of | 0.5 mM Ca | WT | 12 | 5 |
| CA3-CA1 EPSC | 1 mM Ca | WT | 9 | 4 |
| | 2 mM Ca * | WT | 15 | 6 |
| | 3 mM Ca | WT | 6 | 2 |
| | 0.5 mM Ca | KO | 8 | 4 |
| | 1 mM Ca | KO | 7 | 6 |
| | 2 mM Ca * | KO | 10 | 8 |
| | 3 mM Ca | KO | 4 | 2 |
| Figure 3a,b | fEPSP vs. fibre volley | 20-100 uA stimulation | WT | 44 | 11 |
| KO | 25 | 8 |
| Figure 3c,d | Ca\(^{2+}\)-dependence of | 0.5 mM Ca | WT | 4 | 2 |
| CA3-CA1 fEPSP | 1 mM Ca | WT | 11 | 5 |
| | 2 mM Ca * | WT | 11 | 5 |
| | 3 mM Ca | WT | 8 | 3 |
| | 0.5 mM Ca | KO | 4 | 4 |
| | 1 mM Ca | KO | 8 | 4 |
| | 2 mM Ca * | KO | 9 | 5 |
| | 3 mM Ca | KO | 6 | 3 |
| Figure 3e-g | MK801 blockade of NMDAR-fEPSP | 2 mM Ca, single stim | WT | 6 | 2 |
| MK801 blockade of NMDAR-fEPSP | 2 mM Ca, triple stim | WT | 5 | 3 |
| | 2 mM Ca, single stim | KO | 6 | 3 |
| | 2 mM Ca, triple stim | KO | 4 | 3 |
| Extended Data | MK801 blockade of NMDAR-EPSC | 1 mM Ca | WT | 14 | 3 |
| MK801 blockade of NMDAR-EPSC | 2 mM Ca | WT | 11 | 4 |
| | 3 mM Ca | WT | 3 | 2 |
| | 2 mM Ca | KO | 9 | 4 |

*To normalize responses in different Ca\(^{2+}\) concentrations, all Ca\(^{2+}\)-dependence experiments included recordings in 2 mM Ca\(^{2+}\) followed by wash in of different Ca\(^{2+}\) concentrations.