**Short-Term Plasticity at Hippocampal Mossy Fiber Synapses Is Induced by Natural Activity Patterns and Associated with Vesicle Pool Engram Formation**

**Graphical Abstract**

**Highlights**

- Natural activity patterns in hippocampal GCs in vivo induce PTP at mossy fiber synapses
- PTP is primarily caused by an increase in the readily releasable vesicle pool
- PTP is associated with an increase in the number of docked vesicles at active zones
- Sparse activity extends pool engram lifetime, increasing overlap with short-term memory

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**In Brief**

Vandael et al. report that natural activity patterns induce post-tetanic potentiation (PTP) at hippocampal mossy fiber synapses. PTP is primarily caused by an increase in the readily releasable vesicle pool. PTP is associated with an increase in the docked vesicle pool, revealing a structural correlate of presynaptic plasticity.

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Short-Term Plasticity at Hippocampal Mossy Fiber Synapses Is Induced by Natural Activity Patterns and Associated with Vesicle Pool Engram Formation

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SUMMARY

Post-tetanic potentiation (PTP) is an attractive candidate mechanism for hippocampus-dependent short-term memory. Although PTP has a uniquely large magnitude at hippocampal mossy fiber-CA3 pyramidal neuron synapses, it is unclear whether it can be induced by natural activity and whether its lifetime is sufficient to support short-term memory. We combined in vivo recordings from granule cells (GCs), in vitro paired recordings from mossy fiber terminals and postsynaptic CA3 neurons, and “flash and freeze” electron microscopy. PTP was induced at single synapses and showed a low induction threshold adapted to sparse GC activity in vivo. PTP was mainly generated by enlargement of the readily releasable pool of synaptic vesicles, allowing multiplicative interaction with other plasticity forms. PTP was associated with an increase in the docked vesicle pool, suggesting formation of structural “pool engrams.” Absence of presynaptic activity extended the lifetime of the potentiation, enabling prolonged information storage in the hippocampal network.

INTRODUCTION

Short-term memory, a form of memory that covers a timescale of tens of seconds to minutes, is an essential component of several brain functions, from the level of simple reflexes to the level of consciousness and intelligent behavior (Squire and Zola-Morgan, 1991; Goldman-Rakic, 1995). Early work suggested that sustained neuronal activity underlies short-term memory (Goldman-Rakic, 1995). However, more recent studies proposed that short-term memory is held in synapses, suggesting synaptic facilitation as a possible mechanism (Mongillo et al., 2008; Zenke et al., 2015; Jackman and Regehr, 2017). Although the idea that synaptic facilitation underlies short-term memory is appealing, the time course of facilitation is fast, decaying within tens to hundreds of milliseconds, whereas short-term memory extends over a much longer timescale. In the hippocampus, a brain region that contributes to short-term memory, information can be held in the circuit for several minutes during delayed match- or nonmatch-to-sample tasks (Squire and Zola-Morgan, 1991; Deadwyler et al., 1996). Thus, other forms of synaptic plasticity must be involved. A possible candidate might be post-tetanic potentiation (PTP), a form of short-term plasticity that decays within tens to hundreds of seconds (Zucker and Regehr, 2002). Within the hippocampus, PTP shows a unique magnitude at the mossy fiber-CA3 pyramidal neuron synapse, where PTP regulates synaptic strength by nearly an order of magnitude (Griffith, 1990; Langdon et al., 1995; Salin et al., 1996; Toth et al., 2000; Vyleta et al., 2016; reviewed by Nicoll and Schmitz, 2005). However, the physiological significance and the biophysical mechanisms of mossy fiber PTP are largely unknown. Although previous attempts were made to test whether physiological activity patterns induce mossy fiber plasticity (Gundlfinger et al., 2010; Mistry et al., 2011), the results remained inconclusive, because the applied patterns were likely recorded from non-granule cells (Neunuebel and Knierim, 2012). Thus, whether mossy fiber PTP forms the basis of short-term memory in the hippocampus remains unclear.

To address the functional significance, mechanisms, and possible structural correlates of PTP at hippocampal mossy fiber synapses, we combined in vivo recordings from hippocampal granule cells (GCs) in behaving animals, in vitro paired recordings from mossy fiber terminals and postsynaptic CA3 pyramidal neurons (Vyleta et al., 2016), and functional electron microscopy (EM) analysis of mossy fiber synapses (“flash and freeze”; Watanabe et al., 2013a, 2013b; Borges-Merjane et al., 2020). Our results indicate that PTP is induced by physiological GC activity, generated by an increase in the size of the readily releasable vesicle pool (RRP), and associated with formation of structural “pool engrams.”

RESULTS

Superbursts in GCs Induce PTP at Hippocampal Mossy Fiber-CA3 Synapses

To address whether hippocampal mossy fiber PTP is induced by natural activity patterns, we examined the activity of presynaptic dentate gyrus GCs in head-fixed behaving rodents (X.Z.,...
A. Schlögl, and P.J., unpublished data; Figure 1). Simultaneous whole-cell current-clamp and local field potential (LFP) recordings were performed from GCs in the dorsal hippocampus during quiet and running periods (Figure 1A). Analysis of a total population of 43 morphologically identified active GCs revealed that, on average, GCs fired at low frequency (average firing frequency, 0.029 ± 0.011 Hz; mean ± SEM; X.Z.; A. Schlögl, and P.J., unpublished data), consistent with previous immediate-early gene expression and Ca2+ imaging studies (Alme et al., 2010; Pilz et al., 2016). However, interspike intervals (ISIs) were widely distributed, indicating that activity was highly patterned (Figures 1B and 1C). ISI distributions were significantly better fit with the sum of three rather than two log-normal components, suggesting that activity was comprised of single spikes, bursts, and bursts of bursts ("superbursts" [SBs]) (p = 0.047; Figure 1C). On average, SBs contained 9.2 ± 0.5 spikes (Figure 1D) and had a mean duration of 603 ± 41 ms (Figure 1E). SBs were significantly more frequent during running than quiet periods (velocity >2 cm s\(^{-1}\), 0.0197 ± 0.0086 Hz versus 0.0073 ± 0.0053 Hz, p = 0.004; Figure 1F). However, the SB frequency was not significantly different between theta and non-theta epochs (theta-to-delta power ratio in the LFP signal >4, 0.0149 ± 0.0069 Hz versus 0.0101 ± 0.0071 Hz, p = 0.309; Figure 1F). Thus, differences in SB frequency may be explained by direct effects of running rather than indirect effects of increased theta activity. The occurrence of SBs was independent of the location of the GC soma (Figure 1G). SBs occurred in 20 of 43 active GCs (Figure 1H, left). Event-based analysis showed that the majority of events (70.7%) were single spikes, whereas 19.1% were bursts, and 10.1% were SBs (Figure 1H, center). However, spike-based analysis revealed that only 16.4% of spikes occurred in isolation, whereas 38.3% and 45.3% occurred in bursts and SBs, respectively (Figure 1H, right). Thus, our results identify SBs as a prominent form of activity in the dentate gyrus.
High-frequency extracellular stimulation of multiple mossy fiber inputs, typically by trains of 100 pulses at a frequency of 100 Hz (HFS\textsubscript{100APs}), is a widely used paradigm for induction of mossy fiber PTP (Griffith, 1990; Langdon et al., 1995; Salin et al., 1996; Toth et al., 2000). However, our results indicate that the natural activity of GCs is substantially more restricted. First, the low spiking frequency of GCs implies that, among the mossy fiber inputs converging on a single CA3 pyramidal neuron (~50; Amaral et al., 1990), only a single mossy fiber synapse will be active at any point in time. Second, the number of APs per SB is significantly smaller than in the HFS\textsubscript{100APs} protocol. To test whether in vivo-like activity in a single presynaptic terminal is sufficient to induce PTP, we made paired recordings between mossy fiber terminals and postsynaptic CA3 pyramidal neurons (Figure 2). Single presynaptic terminals were minimally invasively stimulated in the tight-seal, cell-attached voltage-clamp configuration (Bischofberger et al., 2006; Vyleta and Jonas, 2014; Vyleta et al., 2016), and excitatory postsynaptic currents (EPSCs) were recorded in postsynaptic CA3 pyramidal neurons in the whole-cell voltage-clamp configuration (Brown and Johnston, 1983; Figure 2A).

To determine the threshold for PTP induction, we applied trains of 3, 9, 18, 36, and 72 stimuli to single presynaptic terminals, all at a frequency of 100 Hz (Figures 2B and 2C). Although trains of three stimuli (HFS\textsubscript{3APs}) did not significantly change the EPSC amplitude, and trains of nine stimuli (HFS\textsubscript{9APs}) had only subtle effects, trains of 18, 36, and 72 stimuli (HFS\textsubscript{18APs}, HFS\textsubscript{36APs}, and HFS\textsubscript{72APs}, respectively) induced robust PTP (300.1% ± 58.1%, 258.9% ± 62.7%, and 668.5% ± 462%, respectively; 9 mossy fiber terminal-CA3 pyramidal neuron pairs; p = 0.007, 0.0117, and 0.0171, respectively; Figure 2D). PTP induced by HFS\textsubscript{72APs} was only slightly different from that induced by HFS\textsubscript{100APs} in isolation (432.5% ± 73.9%; 9 mossy fiber terminal-CA3 pyramidal neuron pairs; p = 0.025), arguing against cumulative effects in the paradigm. Similarly, trains of 18 stimuli induced robust PTP at near-physiological temperature (Figures S1A –S1C).

To quantify the overlap between PTP induction and natural action potential (AP) activity, we plotted the magnitude of PTP against the number of stimuli during induction. We fit the dependence of PTP on AP number with a Hill equation (Figure 2E) and compared the resulting curve with the distribution of the number of APs per SB in vivo (Figure 2F). Consistent with the
physiological significance of PTP, the two curves showed marked overlap. Furthermore, quantitative analysis indicated that SBs recorded in vivo in identified GCs induced PTP with an average magnitude of 129% (range, 113%–176%; Figure 2G). To corroborate this conclusion, we directly applied experimentally recorded SB patterns as stimuli in paired mossy fiber terminal-CA3 pyramidal neuron recordings at near-physiological temperature (Figures 2H and 2I). Although SBs with 10 APs induced small but significant PTP, SBs with 24 APs induced marked PTP (9 pairs, p = 0.02 and 0.007, respectively; Figures 2H and 2I). Similarly, SBs with 24 APs induced robust PTP at near-physiological temperature with a near-physiological extracellular Ca2+ concentration (Rancz et al., 2007; Borst, 2010; Figures S1G–S1I). Taken together, natural activity patterns of identified GCs are sufficient to induce PTP at single hippocampal mossy fiber synapses.

PTP Is Mainly Generated by an Increase in RRP Size

Next we addressed the biophysical mechanisms underlying PTP at hippocampal mossy fiber synapses. In the calyx of Held, a synapse in the auditory brain stem in which PTP has been well characterized, PTP is mainly caused by a rise in the release probability (Pr) for each vesicle (Habets and Borst, 2005; Korogod et al., 2005; Fioravante et al., 2011). However, changes in the size of the RRP have also been reported (Habets and Borst, 2007; Lee et al., 2008, 2010; Chu et al., 2014). To determine the biophysical mechanisms underlying PTP, we analyzed cumulative EPSC peak amplitudes during 50-Hz test trains of 10 stimuli applied every 20 s (Schneggenburger et al., 1999; Neher, 2015), just below the threshold for PTP induction (Figure 3). Cumulative EPSC peak amplitude was plotted against stimulus number, and data were analyzed by linear regression of the last 3–5 data points (Schneggenburger et al., 1999; Neher, 2015). The size of the RRP was determined as the intersection of the regression line with the ordinate, Pr was measured as the ratio of the first EPSC amplitude over RRP size, and the refilling rate was obtained from the slope of the regression line (Figure S2A).

We then measured RRP and Pr before and after PTP induction (Figures 3D–3H). In contrast to our expectations (Habets and Borst, 2005; Korogod et al., 2005), we found that PTP was largely mediated by an increase in RRP size. At a time point 20 s after HFS100APs, the RRP markedly increased from 1048.7 ± 204.4 pA during pre-HFS periods to 2541.4 ± 355.6 pA during post-HFS periods (12 pairs, p = 0.002; Figures 3D and 3E). In contrast Pr increased only slightly, from 0.19 ± 0.03 pre-HFS to 0.24 ± 0.03 post-HFS (p = 0.015; Figures 3D and 3F). Furthermore, the refilling rate during the test train was unchanged (10.9 ± 2.0 pA/s pre-HFS versus 10.3 ± 1.8 pA/s post-HFS, p = 0.38; Figures 3D and 3G). Consistent with the hypothesis that PTP was generated by a change in RRP rather than Pr, PTP was associated with only a nonsignificant decline in the paired-pulse ratio (p = 0.158; Figure 3C). Additionally, in plots of RRP, Pr, and refilling...
rate against experimental time, only the change in RRP closely followed the time course characteristic for PTP (Figure 3H).

To test whether changes in quantal size (Q) contributed to PTP, we measured miniature EPSCs (mEPSCs) before and after PTP induction (Figure 4). After PTP had been induced by HFS100APs in the presynaptic tight-seal, cell-attached stimulation, 1 mM tetrodotoxin (TTX) and 10 mM tetraethylammonium (TEA) were applied to the bath. After transition into the presynaptic whole-cell configuration, low-amplitude pulses were applied to the presynaptic terminal to induce mEPSCs (Figures 4A and 4B). In a time interval 20–60 s after HFS100APs, the mean EPSC peak amplitude was slightly but significantly increased in comparison with later periods (59.9 ± 4.2 pA versus 52.9 ± 3.2 pA, 7 pairs, p = 0.017; Figures 4C and 4D). However, the median EPSC peak amplitude was not significantly changed, indicating an increase in the proportion of larger events (45.7 ± 3.6 pA versus 41.8 ± 3.3 pA, p = 0.12; Figure 4D). Thus, an increase in Q contributes only minimally to hippocampal mossy fiber PTP.

The validity of our analysis was confirmed by several additional tests. First, estimates of RRP were largely independent of the fitting range used for linear regression in the cumulative EPSC analysis (Figures S2B–S2G). Second, similar conclusions were reached in the presence of 1 mM of the low-affinity competitive antagonist kynurenic acid (kyn) receptors confounded our measurements (Habets and Borst, 2005; Figure S3). Third, analysis with an alternative method that assumes that the refilling rate depends on prior release of synaptic vesicles (Thanawala and Regehr, 2013) gave similar conclusions (Figures S4A–S4C). Fourth, similar conclusions were obtained at reduced and increased extracellular Ca²⁺ concentrations (1.2 mM and 4 mM, respectively; Figure S5). Additionally, these results directly demonstrate that PTP-induced changes in RRP are largely independent of Ca²⁺ concentration-related changes in Pr. Finally, analysis with a model containing three vesicle pools (docked, primed, and superprimed pool; Lee et al., 2013; Taschenberger et al., 2016) confirmed that the size of the RRP was increased after PTP (Figures S4D–S4F). Taken together, these results converge on the conclusion that PTP at the hippocampal mossy fiber synapse was primarily mediated by an increase in the size of the RRP.

PTP Is Associated with an Increase in the Docked Vesicle Pool

According to our results, PTP was generated by an increase in RRP size. Such an increase may arise from changes in vesicle docking, priming, or both (Imig et al., 2014). To distinguish
between these possibilities, we performed functional EM (“flash and freeze”) experiments at hippocampal mossy fiber synapses. Thin hippocampal slices were embedded in a sandwich between two sapphire blades, hippocampal GCs were stimulated by light pulses, and slices were subjected to high-pressure freezing at defined time intervals for subsequent EM analysis (Borges-Merjane et al., 2020).

(B) Example transmission EM (TEM) micrographs of active zones of hippocampal mossy fiber synapses in control slices (left), slices frozen immediately after stimulation with 100 light pulses (center), and slices frozen 20 s after stimulation (right). Images were taken from the mossy fiber tract in the stratum lucidum of the CA3 region, primarily CA3b and CA3c. White bars indicate postsynaptic densities. (C–E) Scatterplot (C), cumulative distributions (D), and histograms (E) of the number of docked vesicles per 100 nm of active zone profile length for a non-light stimulated control (black), slices frozen immediately after HFS100APs stimulation at 20 Hz (blue), and slices frozen after HFS100APs stimulation + 20 s recovery time (red). Bars show mean and standard deviation. Each data point in (C) represents an active-zone profile. Note that the number of docked vesicles increased 20 s after HFS, indicating “overfilling” of the docked vesicle pool.

(F–H) Similar plots as shown in (C)–(E) but for diameter of docked vesicles. The diameter of docked vesicles was slightly but significantly larger in the HFS100APs stimulation + 20 s recovery time in comparison with control experiments. Each data point in (F) represents a docked synaptic vesicle. Data for the HFS100APs stimulation (blue) include measurements taken from a previous study (Borges-Merjane et al., 2020).

In (C) and (F), boxes indicate mean values, and error bars denote standard deviation. *p < 0.01, ****p < 0.0001.

Figure 5. Functional EM Analysis Reveals an Increase in the Number of Docked Vesicles during PTP

(A) Left: schematic illustration of “flash and freeze” experiments at hippocampal mossy fiber synapses. Thin hippocampal slices were embedded in a sandwich between two sapphire blades, hippocampal GCs were stimulated by light pulses, and slices were subjected to high-pressure freezing at defined time intervals for subsequent EM analysis (Borges-Merjane et al., 2020).

(B) Example transmission EM (TEM) micrographs of active zones of hippocampal mossy fiber synapses in control slices (left), slices frozen immediately after stimulation with 100 light pulses (center), and slices frozen 20 s after stimulation (right). Images were taken from the mossy fiber tract in the stratum lucidum of the CA3 region, primarily CA3b and CA3c. White bars indicate postsynaptic densities.
high-pressure freezing and subsequently processed for EM analysis (Figures 5A and S6). Mossy fiber terminals were identified based on location in the stratum lucidum, large size, termination on thorny ex crescences of CA3 pyramidal cells, and high density of synaptic vesicles (Chicurel and Harris, 1992; Rollenhagen et al., 2007; Borges-Merjane et al., 2020).

In unstimulated control slices, the number of docked vesicles (within 5 nm of the plasma membrane) was 1.16 ± 0.04 per 100-nm active zone profile length (mean ± standard deviation, 154 profiles, 4 mice; Figures 5B–5E), consistent with previous measurements (Rollenhagen et al., 2007). When slices were frozen immediately after HFS100APs, the number of docked vesicles per profile was strongly reduced to 0.33 ± 0.03 per 100-nm active zone profile length (p < 0.0001, 234 profiles, 3 mice; Figures 5C and 5D, blue symbols and lines), as reported previously (Borges-Merjane et al., 2020). This was consistent with a model in which synaptic vesicles at hippocampal mossy fiber synapses underwent full fusion. When slices were frozen 20 s after HFS100APs, the number of docked vesicles was 1.45 ± 0.04 per 100-nm active zone profile length (193 profiles, 4 mice; Figures 5C–5E, red symbols and lines). Thus, the number of docked vesicles per profile was not only markedly higher than directly after HFS (p < 0.0001) but also significantly larger than under control conditions (p = 0.003; Figures 5C–5E).

On average, the number of docked vesicles per profile 20 s after HFS was 1.243 times larger than in interleaved control experiments. Because the number of vesicles per active zone (measured in 2 dimensions) is quadratically related to the number of vesicles per profile (measured in 1 dimension; Figure S6D), we estimate that the number of docked vesicles per active zone was increased by a factor of (1.243)² = 1.545. To further corroborate that PTP was evoked in our “flash and freeze” experiments, we measured the effects of optogenetic HFS100APs, at 20 Hz in electrophysiological experiments under identical conditions (Figures S6A and S6B). Optogenetic HFS100APs induced significant PTP (5 CA3 pyramidal neuron recordings), although the magnitude was smaller (207% ± 39%) than with electrical HFS100APs (432% ± 73%, p = 0.02; Figure 3B). Taken together, the increase in the RRP after HFS (Figure 3) can be largely explained by an increase in the number of docked vesicles.

Detailed analysis of electron micrographs revealed that the mean diameter of docked vesicles was also slightly but significantly increased 20 s after HFS, from 37.7 ± 0.8 nm under control conditions to 43.8 ± 1.1 nm 20 s after HFS (Figures 5F–5H). Because larger vesicles might release a higher amount of glutamate, this may explain the additional increase in mean mEPSC amplitude observed in our paired recording experiments (Figure 4D). Thus, HFS100APs led to a marked increase in the number of docked vesicles and a slight increase in their mean diameter.

Pool Refilling Determines PTP
PTP increased the size of the RRP, implying that pool refilling is a major determinant of short-term plasticity. To further test this hypothesis, we reciprocally examined the relation between refilling rate and PTP (Figure 6). First, we tested whether pharmacological manipulations that affected PTP also changed the refilling rate. Because PTP at the hippocampal mossy fiber synapse is known to involve the cyclic AMP (cAMP)-protein kinase A (PKA) pathway (Weisskopf et al., 1994; Alle et al., 2001), we examined the effects of the PKA blocker H-89 (Figures 6A–6C). Surprisingly, we found that 10 μM H-89 reduced the EPSC amplitude under control conditions from 342.3 ± 33.6 pA to 61.6 ± 21.6 pA after 380 s of application of the drug (p = 0.0179; Figures 6A and 6B). As expected, subsequent HFS100APs applied in the presence of H-89 had no additional effects on EPSC amplitude (p = 0.176; Figures 6A and 6B). These results suggest that PKA regulates basal transmission and PTP induction. To determine the underlying mechanisms, we analyzed cumulative EPSC amplitude during HFS100APs under control conditions and in the presence of H-89 (Figures 6C and 6E). H-89 only slightly reduced the size of the RRP (from 1,862.7 ± 407.3 pA to 1,599 ± 294 pA) and left Pr unchanged (0.104 ± 0.031 versus 0.086 ± 0.030, 7 pairs, p = 0.50). However, H-89 substantially decreased the refilling rate during HFS100APs (from 7.97 ± 1.27 pA·ms⁻¹ to 2.53 ± 0.62 pA·ms⁻¹, p = 0.004; Figures 6C and 6E). Thus, PKA block abolished PTP and, in parallel, reduced the pool refilling rate during HFS100APs.

Next we examined whether the pharmacological manipulations that affected refilling also changed PTP. At the calyx of Held and cerebellar parallel fiber-interneuron synapses, pool refilling is dependent on an intact actin cytoskeleton (Sakaba and Neher, 2003; Miki et al., 2018). Thus, we tested the effects of the actin polymerization inhibitors latrunculin B and cytochalasin D (Figures 6D–6G). As expected from the results from the calyx of Held, 20 μM latrunculin B and 10 μM cytochalasin D reduced refilling rate during HFS100APs from 7.97 ± 1.27 pA·ms⁻¹ to 4.3 ± 0.84 pA·ms⁻¹ (p = 0.025; Figures 6D and 6E). In parallel, latrunculin B and cytochalasin D attenuated PTP from 432% ± 74% under control conditions to 136% ± 38% in the presence of polymerization inhibitors (p = 0.003, 8 pairs; Figures 6F–6H). To further test the hypothesis that PTP is generated by pool refilling during HFS, we plotted the magnitude of PTP after HFS100APs for all datasets (under control conditions, in the presence of H-89, and in the presence of latrunculin B and cytochalasin D) against the refilling rate during HFS100APs (Figure 6I). The magnitude of PTP was highly correlated with the refilling rate during HFS100APs (Spearman rank correlation coefficient ρ = 0.61, p = 0.0005, 28 pairs; Figure 6I). Taken together, these results corroborate the hypothesis that vesicle pool refilling underlies PTP. This conclusion was further supported by our three-pool model with activity-dependent refilling, which closely replicated our experimental datasets before, during, and after PTP induction (Figures S4D–S4F). In summary, these results indicate that PTP is generated by enhanced vesicle refilling during HFS.

Extended Time Course of Potentiation in Delay Paradigms
If PTP has physiological significance, then conditions for induction and readout must be satisfied. Thus, single spikes or bursts must follow SBs and fall into the time window of PTP. To test whether this condition occurs, we quantified the likelihood of SB-single spike and SB-burst sequences in our in vivo dataset (Figures 7A–7E). SBs were more frequently followed by a single spike (55%) than by a burst (10%) or a second SB (30%) (Figure 7A). The latency between an SB and subsequent events,
quantified on a cell-by-cell basis, was 6.0 ± 2.1 s for the first AP and 91.7 ± 34.1 s for the last AP (20 GCs; Figures 7B–7E). Thus, although the range of SB-AP intervals overlapped with the time window of PTP, a substantial proportion of latencies extended beyond this time window.

How could these late events be used for PTP readout? If PTP is mediated by an increase in the size of vesicle pools, then exocytosis may dissipate the potentiation. Conversely, the absence of activity might extend the lifetime of the “pool engram.” To test this prediction, we compared PTP with two experimental
protocols: the standard protocol, in which test pulses directly followed HFS100APs with a 20 s repetition rate, and an alternative protocol, in which test pulses followed HFS100APs after a delay of 1–5 min (Figure 7F). Lack of activity during the delay period was verified by the absence of action currents in the presynaptic terminal. For the standard protocol, PTP had a magnitude of 241% and decayed with a time constant of 81 s. If PTP decayed with uniform time course after induction, then it should have decayed to 107% after a 4-min delay period. However, in contrast to this prediction, we found that PTP after 3–4 min was similar to that probed immediately after HFS100APs (245% ± 23% versus 241% ± 31%, 6 pairs, p = 0.97; Figures 7F and 7G). After the delay, the selective reliance of PTP on RRP size changes (Figure 3) was further enhanced, with significant changes in RRP but no changes in Pr (Figures 7H and 7I). Thus, the time course of PTP was extended in the complete absence of presynaptic activity. The sparse and patterned activity of GCs is ideally suited to use this mechanism. Although the generation of SBs guarantees efficient storage of information, the long SB-AP time interval enables reliable readout (Figure 7J).

**DISCUSSION**

Our results provide compelling evidence of physiological relevance of PTP. First, in vivo recordings identify SBs as a novel form of patterned activity of GCs. Second, PTP at hippocampal mossy fiber synapses shows a low induction threshold. Thus, the PTP induction curve and the distribution of APs in SBs overlap. Finally, SBs are often followed by single APs. Taken together, the natural activity patterns of GCs are not only highly suitable for induction of PTP but also for efficient readout.

Our functional analysis shows that PTP is mediated by enlargement of the RRP (Figure S7A). This implies that PTP requires pool refilling during or after HFS. This hypothesis is further corroborated by the effects of PKA blockers and actin polymerization inhibitors, which reduce refilling and PTP in parallel. An attractive molecular mechanism that links the effects of PKA and actin may involve synapsins. In this model, phosphorylation may enhance pool refilling by dissociating synaptic vesicles from the actin cytoskeleton (Gitler et al., 2008; Patzke et al., 2019; reviewed by Cingolani and Goda, 2008). A large reserve pool may
be needed to ensure fast and sustained reloading of the RRP at multiple active zones. Thus, the model attributes a function to the large vesicle pool in mossy fiber terminals (Chicurel and Harris, 1992; Rollenhagen et al., 2007), which has puzzled researchers for decades.

Although PTP predominantly increases the RRP, other forms of mossy fiber plasticity are well known to change Pr (that is, the fusion probability per vesicle) through AP broadening (Geiger and Jonas, 2000), buffer saturation (Vyleta and Jonas, 2014), and tightening of Ca\(^{2+}\) channel-sensor coupling (Midorikawa and Sakaba, 2017; Jackman and Regehr, 2017; Figure S7B). Thus, the mossy fiber synapse is endowed with orthogonal forms of plasticity, which may interact multiplicatively. However, this scheme is a simplification because, according to our results, multiple mechanisms contribute to PTP (increase in RRP, Pr, and Q). Thus, changes in RRP and Q representing a major component of PTP will multiplicatively interact with changes in Pr, reflecting a minor component of PTP and other plasticity forms. In combination, these changes enrich the repertoire of single-synapse computations (Abbott and Regehr, 2004; Silver, 2010; Henze et al., 2002; Vyleta et al., 2016; Chamberland et al., 2018). Although an increase in Pr may lead to a burst-to-spike transmission mode, an increase in RRP could facilitate a burst-to-burst transmission regime (Vyleta et al., 2016). Such a transmission mode may explain the prevalence of bursting in CA3 pyramidal neurons in vivo (Kowalski et al., 2016).

Our results reveal that structural changes are associated with short-term plasticity. Although structural changes during presynaptic plasticity have been reported in invertebrate synapses (for example, Aplysia; Bailey et al., 2015), such changes have not been identified in mammalian synapses. It is widely thought that PTP is caused by an increase in Ca\(^{2+}\) concentration and an associated increase in Pr (Regehr et al., 1994; Zucker and Regehr, 2002; Habets and Borst, 2005; Korogod et al., 2005). However, such changes may be too short-lasting to contribute to short-term memory. Our functional EM (“flash and freeze”) experiments reveal that the number of docked vesicles increases 20 s after HFS\(_{100\text{Hz}}\) stimulation at 20 Hz. In the absence of activity, the increase in the docked vesicle pool seems to have a markedly prolonged lifetime. This may be related to the low rates of spontaneous release and vesicle undocking (Murthy and Stevens, 1999). The long lifetime of the pool engram may be explained by the energetics of the SNAP receptor (SNARE) complex (consisting of syntaxin, SNAP-25, and synaptobrevin). In docked or primed vesicles, SNARE complexes may be partially zipped, leading to an almost irreversible reaction toward exocytosis (Fasshauer et al., 2002). This suggests that the SNARE complex not only provides the energy for membrane fusion but also implements a molecular mechanism for information storage. Thus, exocytosis and synaptic plasticity might be more intimately linked than previously thought.

Our findings suggest that PTP mediated by a vesicle pool mechanism is suitable for short-term storage of information in the hippocampus (Squire and Zola-Morgan, 1991). First, PTP shows a long decay time course that is further extended in the absence of activity. Additionally, the time course of potentiation may be prolonged by neuromodulators (Huang and Kandel, 1996). Thus, the time course of PTP approaches that of hippocampal short-term memory (Squire and Zola-Morgan, 1991; Deadwyler et al., 1996). Second, the pool mechanism of PTP is energetically efficient. Although the maintenance of enlarged vesicle pools does not consume any energy, persistent AP generation requires an ongoing energy supply (Attwell and Laughlin, 2001). Third, pool mechanisms may be particularly suitable for storage of information in the dentate gyrus-CA3 circuit, in which sparse activity prevails (Pernia-Andrade and Jonas, 2014; Pilz et al., 2016; X.Z., A. Schölgl, and P.J., unpublished data; Figure 1). Pool mechanisms may be particularly useful during delayed nonmatch-to-sample tasks, in which neurons often show high activity in sample or test phase but low activity in the delay phase (Deadwyler et al., 1996; Wiebe and Staubli, 1999).

Classical work tried to identify engrams; that is, physical or chemical changes underlying the formation of new memories (Lashley, 1950). Does the increase in the number of docked vesicles in presynaptic terminals represent a “vesicle pool engram”? It is well established that dentate gyrus GCs participate in formation of engrams and that their reactivation is sufficient to trigger memory recall and behavioral responses (Liu et al., 2012). It has been proposed that hippocampal engram cells show enhanced excitability and a higher functional connectivity than nonengram cells (Josselyn and Tonegawa, 2020). However, the underlying physical or chemical modifications remain elusive. Our findings suggest that changes in the docked vesicle pool in mossy fiber terminals may contribute to formation of engrams. Although our results demonstrate that the PKA pathway regulates vesicle pool size, previous work indicated a link between the PKA pathway in GCs and contextual memory formation (Jones et al., 2016). Taken together, these results may suggest an intriguing connection between synaptic vesicle pools and hippocampal memory. More work is needed to rigorously test the vesicle pool engram hypothesis and close the gap between synaptic and behavioral level.

**STAR+METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead Contact
  - Materials Availability
  - Data and Code Availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Animal experiments
- **METHOD DETAILS**
  - In vivo recordings from identified dentate gyrus GCs
  - Brain slice preparation
  - Presynaptic cell-attached and whole-bouton recording
  - Postsynaptic whole-cell somatic recording
  - PTP induction protocols
  - Thin-slice preparation for functional electron microscopy
  - High-pressure freezing
  - Freeze substitution, embedding, and electron microscopy
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.neuron.2020.05.013.

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AUTHOR CONTRIBUTIONS

D.V. performed the in vitro paired recordings. C.B.-M. carried out the “flash and freeze” experiments. X.Z. conducted the in vivo recordings. P.J. performed modeling and wrote the paper. All authors analyzed the data and jointly revised the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, Peptides, and Recombinant Proteins** |
| Tamoxifen | Sigma-Aldrich | Cat # T5648-1G |
| Corn Oil | Sigma-Aldrich | Cat # C8267-500 ml |
| NaCl | VWR (Merck) | Cat # 1.06404.1000 |
| Sucrose | Sigma-Aldrich | Cat # 16104 |
| NaHCO3 | VWR (Merck) | Cat # 1.06329.1000 |
| D-glucose | VWR (Merck) | Cat # 1.08342.1000 |
| KCl | VWR (Merck) | Cat # 26764.232 |
| Na2HPO4 | VWR (Merck) | Cat # 1.06580.0500 |
| NaH2PO4 | VWR (Merck) | Cat # 1.06346.0500 |
| CaCl2 | VWR (Merck) | Cat # 1.02382.0250 |
| MgCl2 | Honeywell | Cat # M9272-1KG |
| HEPES | Sigma-Aldrich | Cat # M3375-100G |
| EGTA | Sigma-Aldrich | Cat # EO396-100G |
| Na2ATP | Sigma-Aldrich | Cat # A3377-100G |
| Polyvinylpyrrolidone (PVP) | Sigma-Aldrich | Cat # PVP10-100G |
| Sodium azide | Sigma-Aldrich | Cat # S8032-25G |
| Osmium tetroxide | Science services (EMS) | Cat # 19130 |
| Durcupan ACM Resin Single component A | Sigma-Aldrich | Cat # 44611-100 ml |
| Durcupan ACM Resin Single component B | Sigma-Aldrich | Cat # 44612-100 ml |
| Durcupan ACM Resin Single component C | Sigma-Aldrich | Cat # 44613-100 ml |
| Durcupan ACM Resin Single component D | Sigma-Aldrich | Cat # 44614-100 ml |
| Uranyl acetate 2 H2O | Serva | Cat # 77870.02 |
| Lead (II) nitrate | Sigma-Aldrich | Cat # 228621-100G |
| Acetone, HPLC grade | ChemLab | Cat # CL00.0172.2500 |
| Ketamine | Intervet | Z.Nr. 8-00335 100 mg/ml |
| Xylazine | Graeub | Z.Nr. 8-00178 20 mg/ml |
| Lidocaine | Sigma | L-1026-1VL |
| Dexamethasone ointment | Bayer | Cat # PZN 0829388 |
| Cyanoacrylate superglue | Uhu | Cat # 45570 3 g |
| Metacam | Boehringer | 2 mg/ml |
| Silicone elastomer | Kwik-cast, World Precision Instruments | N/A |
| Potassium D-Gluconate | Sigma-Aldrich | Cat # G4500-100 g |
| NaGTP | Sigma-Aldrich | Cat # G8877-250 mg |
| Biocytin | Molecular probes | Cat # B1592 |
| Paraformaldehyde | TAAB | Cat # FO 17/1 |
| Glutaraldehyde | Carl Roth | Cat # 4157.1 |
| Saturated picric acid solution | Sigma-Aldrich | Cat # P6744-1GA |
| Triton X-100 | Sigma-Aldrich | Cat # X100-100ml |
| Avidin-biotinylated horseradish peroxidase complex | ABC, Vector Laboratories | Cat # PK5100 |
| 3,3’-Diaminobenzidine tetrahydrochloride | Sigma-Aldrich | Cat # D5637-5 g |
| Cobalt | Sigma-Aldrich | Cat # C8661-25 g |
| Nickel (II) chloride hexahydrate | Sigma-Aldrich | Cat # 223387-25 g |

(Continued on next page)
RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Peter Jonas (peter.jonas@ist.ac.at).

Materials Availability
This study did not generate new unique reagents.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mowiol 4-88         | Carl Roth | Cat # 713.2 |
| Cytochalasin D      | Tocris | Cat # 1233 |
| Latrunculin B       | Tocris | Cat # 3974/1 |
| H-89 dihydrochloride| Tocris | Cat # 2910/10 |
| Tetrodotoxin citrate| Tocris | Cat # 1069/1 |
| Tetraethylammonium chloride | Sigma-Aldrich | Cat # T2265 |
| Cesium chloride     | Sigma-Aldrich | Cat # C3011-25 g |
| H2O2                | Sigma-Aldrich | Cat # 95321-100 ml |
| Tetraethylammonium chloride | Sigma-Aldrich | Cat # 252859-100 g |
| Glycerol            | Sigma-Aldrich | Cat # G9012-500 ml |
| HCl                 | Merck | 1.09057.1000 |

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Borosilicate glass (2 mm outside/ 1 mm inside) | Hilgenberg | Cat# 1907542 |

Experimental Models: Organisms/Strains

| Prox1-CreERT2 mouse line | Bazigou et al., 2011 | PMID: 21765212 |
|--------------------------|----------------------|-----------------|
| C57BL6/J wild-type mice  | Charles River Germany (from The Jackson Laboratory) | RRID:IMSR_JAX:000664 |
| B6;129S-Gt(ROSA)26Sortm32(CAG-COPA-H134R/EYFP)Hze/J mouse line | The Jackson Laboratory | RRID:IMSR_JAX:012569 |
| Wistar Rats              | Preclinical facility, IST Austria Janvier Labs | RRID:RGD_13508588 |

Software and Algorithms

| Multiclamp (version 1.3.0.05) | Axon Instruments/Molecular Devices | https://www.moleculardevices.com/ |
|-------------------------------|-----------------------------------|----------------------------------|
| Igor Pro (version 6.3.4.1)    | WaveMetrics | https://www.wavemetrics.com/ |
| Radius Software               | EMSIS | https://www.emsis.eu/products/software/radius/ |
| Stimfit (version 0.14.14)     | Guzman et al., 2014 | PMID: 24600389 |
| AxoGraph (version 1.7.2)      | AxoGraph | https://axograph.com/ |
| Fiji (ImageJ version 2.0.0-rc-69/1.52n) | NIH, Open source | https://fiji.sc/ |
| Graphpad Prism (version 8)    | Graphpad | https://www.graphpad.com/ |
| Adobe Illustrator (version 23.0.4) | Adobe | https://www.adobe.com/products/illustrator.html |
| HEKA Patchmaster acquisition software (2x90.1) | HEKA | https://www.heka.com/ |
| Coreldraw X8                  | Coreldraw | https://www.coreldraw.com/en/ |
| MATLAB 2016, 2018             | MathWorks | https://www.mathworks.com/ |
| Coreldraw 2018                | Coreldraw | https://www.coreldraw.com/en |
| Origin 2018                   | OriginLab | https://www.originlab.com/ |
| Python 2.7                    | Python | https://www.python.org/ |
| Mathematica 12.0.0.0          | Wolfram Research | https://www.wolfram.com/mathematica/ |

Other

| RESOURCE AVAILABILITY |
|-----------------------|

Neuron 107, 509–521.e1–e7, August 5, 2020 e2
**Data and Code Availability**
Original data, analysis programs, and computer code were stored in the scientific repositories of the Institute of Science and Technology Austria and are available upon reasonable request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Animal experiments**
Whole-cell patch-clamp recordings in vivo were performed in 35- to 63-day-old C57BL/6 mice (RRID:IMSR_JAX:000664). Paired pre- and postsynaptic recordings in vitro were carried out on 19- to 23-day-old Wistar rats (RRID:RGD_13508588; weight: 55–65 g). Functional electron microscopy (“flash and freeze”) experiments were preformed using Prox1-creER\textsuperscript{T2} (PMID: 21765212; Bazigou et al., 2011) x Ai32 B6;129S-Gt(Rosa)26Sortm32(CAG-COPA*H124R/EYFP)Hze/J (Jackson Laboratory; RRID:IMSR_JAX:012569) crossed mice (Borges-Merjane et al., 2020). Animals were housed under a reversed light cycle (dark: 7:00 am – 7:00 pm, light: 7:00 pm – 7:00 am). For experiments, both male and female animals were used. Experiments were carried out in strict accordance with institutional, national, and European guidelines for animal experimentation, and approved by the Bundesministerium für Wissenschaft, Forschung und Wirtschaft of Austria (A. Haslinger, Vienna; BMWFV-66.018/0007-WF/II/3b/2014, BMWFV-66.018/010-WF/V/36/2015, and BMWFV-66.018/0008-V/3b/2018).

**METHOD DETAILS**

**In vivo recordings from identified dentate gyrus GCs**
To characterize AP frequency, timing, and pattern in morphologically identified GCs, we analyzed a previously acquired dataset from 43 active neurons (K.Z., A. Schölgl, and P.J., unpublished data). Whole-cell patch-clamp recordings in vivo were performed in 35- to 63-day-old C57BL/6 mice of either sex (RRID:IMSR_JAX:000664). Animals were head-fixed, but able to run on a linear belt (Royer et al., 2012; Bittner et al., 2015). Head-bar implantation and craniotomy were performed under anesthesia by intraperitoneal injection of 80 mg kg\textsuperscript{-1} ketamine (Intervet) and 8 mg kg\textsuperscript{-1} xylazine (Graeub), followed by local anesthesia with lidocaine. A custom-made steel head-bar was attached to the skull using superglue and dental cement. After 1 week of recovery, animals were mildly water-restricted for 1 week (2 mL per day), and trained to run on the linear belt for 7–10 days. Water rewards were given when the animal ran over a distance of 180 cm.

The day before recording, two small craniotomies (∼0.5 mm in diameter), one for the patch electrode and one for the LFP electrode, were drilled at the following coordinates: approximately –2.0 mm and –2.5 mm antero-posterior (whole-cell recording and LFP, respectively; measured from bregma), and ~1.2 mm medio-lateral (measured from midline). The dura was left intact, and craniotomies were covered with silicone elastomer (Kwik-Cast, World Precision Instruments). Pipettes were fabricated from borosilicate glass capillaries (1.75 mm outer diameter, 1.25 mm inner diameter). Long-taper whole-cell patch electrodes (9–12 M\textsubscript{Ω}) were filled with a solution containing: 130 mM K-gluconate, 2 mM KCl, 2 mM MgCl\textsubscript{2}, 2 mM Na\textsubscript{2}ATP, 0.3 mM NaGTP, 10 mM HEPES, 18 mM sucrose, 10 or 0.1 mM EGTA, and 0.3% biocytin (pH adjusted to 7.28 with KOH). LFP electrodes (4–6 M\textsubscript{Ω}) were filled with artificial cerebrospinal fluid (ACSF) solution. Whole-cell patch electrodes were advanced through the cortex with 500–600 mbar of pressure to prevent the electrode tip from clogging. After passing the hippocampal CA1 subfield, the pressure was reduced to 20 mbar. After a blind whole-cell recording was obtained, series resistance was calculated by applying a test pulse (+50 mV and –50 mV) to the electrode. Series resistance of the LFP electrode was calculated by applying a test pulse (+50 mV and –10 mV) under voltage-clamp conditions. Recordings were immediately discarded if series resistance exceeded 80 M\textsubscript{Ω}. Bridge balance and pipette capacitance compensation was enabled throughout the recording.

Recordings were performed in the current-clamp configuration without current injection using a HEKA EPC double amplifier. Signals were low-pass filtered at 10 kHz (Bessel) and sampled at 25 kHz with Heka Patchmaster acquisition software. At the end of each recording, the patch pipettes were slowly withdrawn to form an outside-out patch, verifying the integrity of the seal.

APs were detected when the derivative of membrane potential signal was > 50 V s\textsuperscript{-1}. ISI distributions were fit with two or three Gaussian components using a maximum-likelihood procedure. To assess statistical significance, data were analyzed by bootstrap procedures (Efron and Tibshirani, 1998). Original datasets were duplicated 1000 times, randomly permuted, and cut into blocks of original size. Log-likelihood ratios of original data were compared against values of bootstrap replications. The LFP signal was de-trended and down-sampled to 2.5 kHz. Then, the LFP signal was band-pass filtered in the delta (2–4 Hz), theta (5–10 Hz), and gamma range (30–80 Hz), respectively. Hilbert transformation was applied to each signal to extract information about power and phase. Theta and delta power were smoothed using a 2 s sliding window. Epochs were classified as theta if the mean theta-delta power ratio within 2-s time windows was > 4 (Klausberger et al., 2003).

**Brain slice preparation**
Transverse hippocampal slices (350–400 μm thick) were prepared from 19- to 23-day-old Wistar rats of either sex (RRID:RGD_13508588; weight: 55–65 g) according to previously established protocols (Bischofberger et al., 2006). This age and weight range was chosen because morphological properties of mossy fiber synapses were largely mature, while paired recording experiments became increasingly difficult at later stages. Rats were kept under a heating lamp for 1 hour prior to dissection. Animals were lightly anesthetized by isoflurane and killed by rapid decapitation, in accordance with institutional, national, and European
guidelines. Slices were cut from the right hemisphere in ice-cold, sucrose-containing extracellular solution using a vibratome (VT1200, Leica Microsystems), incubated in a maintenance chamber at ~33°C for 45 min for recovery, and subsequently stored at room temperature. High-sucrose ACSF used for cutting contained 64 mM NaCl, 25 mM NaHCO3, 2.5 mM KCl, 1.25 mM NaH2PO4, 10 mM glucose, 120 mM sucrose, 0.5 mM CaCl2, and 7 mM MgCl2. High-sucrose ACSF used for storage contained 87 mM NaCl, 25 mM NaHCO3, 2.5 mM KCl, 1.25 mM NaH2PO4, 10 mM glucose, 75 mM sucrose, 0.5 mM CaCl2, and 7 mM MgCl2, equilibrated with 95% O2 and 5% CO2, ~325 Osm. Experiments were performed at room temperature (24.1 ± 0.2°C; range: 21–26°C), and, in a subset of measurements, at near-physiological temperature (~32°C; range: 29.4–34.0°C; Figure S1). Before onset of the experiment, slices were placed in the recording chamber (~1 mL volume) and superfused with ACSF (125 mM NaCl, 25 mM NaHCO3, 2.5 mM KCl, 1.25 mM NaH2PO4, 2 mM CaCl2, and 1 mM MgCl2) for at least 15 minutes before recording.

Presynaptic cell-attached and whole-bouton recording
Subcellular patch-clamp recordings from mossy fiber boutons and simultaneous recordings from pyramidal neurons in the CA3b,c region of the hippocampus were performed under visual control (Bischofberger et al., 2006; Vyleta and Jonas, 2014). Presynaptic recording pipettes were fabricated from borosilicate glass capillaries (2.0 mm outer diameter, 1.0 mm inner diameter), and had open-tip resistances of 10–20 MΩ. For tight-seal, bouton-attached stimulation under voltage-clamp conditions, the presynaptic pipette contained a K+-based intracellular solution (130 mM K-gluconate, 2 mM KCl, 2 mM MgCl2, 2 mM Na2ATP, 10 mM HEPES, 10 mM EGTA, pH adjusted to 7.3 with KOH, 297–300 Osm), allowing us to measure bouton properties in both cell-attached and subsequent whole-bouton configuration. The presynaptic holding potential was set at ~70 mV to minimize the holding current (less than ~10 pA in all pairs; Alcami et al., 2012). APs in mossy fiber boutons were evoked by brief voltage pulses (amplitude 800 mV, duration 0.1 ms). In the absence of stimulation, action currents were not observed, indicating that bouton-attached stimulation permitted the reliable and precise control of activity in a single presynaptic input. Boutons had diameters of ~2–5 μm, in agreement with the previously reported range of diameters of mossy fiber boutons in light and electron microscopy studies (Chicurel and Harris, 1992; Acsády et al., 1998; Rollenhagen et al., 2007; Borges-Merjane et al., 2020). Stimuli (10 stimuli at 50 Hz) were delivered once every 20 s (i.e., at 0.05 Hz). For mEPSC recordings (Figure 4), a cesium-based internal solution (145 mM CsCl, 2 mM MgCl2, 2 mM Na2ATP, 0.3 mM Na3GTP, 5 mM Na2phosphocreatine, 10 mM EGTA, and 10 mM HEPES, pH was adjusted to 7.3 with CsOH) was used. The presynaptic terminal was held in the whole-cell voltage-clamp configuration, and mossy fiber mEPSCs were evoked by depolarizing the terminal for 4 s to ~10 mV.

Postsynaptic whole-cell somatic recording
Postsynaptic recording pipettes were fabricated from borosilicate glass tubing (2.0 mm outer diameter, 1.0 mm inner diameter), and had open-tip resistances of 3–7 MΩ. They contained an internal solution with 130 mM K-gluconate, 20 mM KCl, 2 mM MgCl2, 2 mM Na2ATP, 10 mM HEPES, and 10 mM EGTA (pH adjusted to 7.28 with KOH, 312–315 Osm), allowing us to measure bouton properties in both cell-attached and subsequent whole-bouton configuration. The presynaptic holding potential was set at ~70 mV to minimize the holding current (less than ~10 pA in all pairs; Alcami et al., 2012). APs in mossy fiber boutons were evoked by brief voltage pulses (amplitude 800 mV, duration 0.1 ms). In the absence of stimulation, action currents were not observed, indicating that bouton-attached stimulation permitted the reliable and precise control of activity in a single presynaptic input. Boutons had diameters of ~2–5 μm, in agreement with the previously reported range of diameters of mossy fiber boutons in light and electron microscopy studies (Chicurel and Harris, 1992; Acsády et al., 1998; Rollenhagen et al., 2007; Borges-Merjane et al., 2020). Stimuli (10 stimuli at 50 Hz) were delivered once every 20 s (i.e., at 0.05 Hz). For mEPSC recordings (Figure 4), a cesium-based internal solution (145 mM CsCl, 2 mM MgCl2, 2 mM Na2ATP, 0.3 mM Na3GTP, 5 mM Na2phosphocreatine, 10 mM EGTA, and 10 mM HEPES, pH was adjusted to 7.3 with CsOH) was used. The presynaptic terminal was held in the whole-cell voltage-clamp configuration, and mossy fiber mEPSCs were evoked by depolarizing the terminal for 4 s to ~10 mV.

PTP induction protocols
Data were acquired with a Multiclamp 700A amplifier, low-pass filtered at 10 kHz, and digitized at 100 kHz using a CED power1401 mkII interface (Cambridge Electronic Design, Cambridge, UK). Pulse generation and data acquisition were performed using FPulse version 3.3.3 (U. Fröbe, Freiburg, Germany). To induce PTP, an HFS100APs stimulation paradigm (100 stimuli at 100 Hz in tight-seal cell-attached mode) was applied to the mossy fiber terminal. In experiments in which the PTP induction threshold was measured (Figures 2B–2G), HFS1APs, HFS3APs, HFS7APs, HFS10APs, HFS20APs, and HFS24APs paradigms (all at 100 Hz) were delivered to the presynaptic terminal. In experiments with physiological activity patterns (Figures 2H and 2I; Figures S1G–S1I), SBS with 10 and 24 APs taken from 107 mossy fiber terminal–CA3 pyramidal neuron recordings were applied. The postsynaptic CA3 pyramidal neuron was recorded under voltage-clamp conditions throughout. In experiments in which mEPSCs were recorded after PTP induction (Figure 4), PTP was induced by HFS100APs in the presynaptic cell-attached configuration, followed by rapid bath application of 1 μM TTX + 10 mM TEA, transition into the presynaptic whole-cell configuration, and subthreshold terminal depolarization to ~10 mV. The first depolarizing pulse was applied ~20 s after HFS.
**Thin-slice preparation for functional electron microscopy**

“Flash and freeze” experiments were performed as previously described (Watanabe et al., 2013a, 2013b; Borges-Merjane et al., 2020). To enable optogenetic GC stimulation, Prox1-CreERT2 (PMID: 21765212; Bazigou et al., 2011) x Ai32 B6;129S-Gt(ROSA) 26Sortm32(CAG-COPA*H124R/EYFP);Hz/J (Jackson Laboratory; RRID:IMSR_JAX:012569) crossed mice were used. For maximum expression of channelrhodopsin in GCs, each mouse received two intraperitoneal (iP) injections of tamoxifen in corn oil (Sigma; 100 mg x kg−1 of mouse body weight) given 2–3 days apart. This procedure resulted in maximal labeling of GCs (Borges-Merjane et al., 2020). Mice were used for experiments after a minimum of 7–10 days after injections. Horizontal hippocampal slices were sectioned at 200 μm thickness for high-pressure freezing and 300 μm thickness for electrophysiology control experiments (Figure S6B) using a vibratome (VT1200S, Leica Microsystems) in ice-cold high-sucrose ACSF. Slices were recovered in high-sucrose ACSF at 35°C for 30–45 min for recovery, transferred to ACSF solution identical to recording solution, and kept at room temperature (~23°C) during storage for up to 4 hours. Finally, slices were heated to physiological temperature (37°C) for 5–10 minutes directly before freezing.

**High-pressure freezing**

High-pressure freezing was performed with a Leica EM ICE high-pressure freezing apparatus equipped with a blue LED light stimulation module (460 nm wavelength; light intensity at specimen 5.5–8.0 mW mm−2). After slicing and recovery, slices were mounted in a sapphire-metal ring sandwich (Figure 5A; Borges-Merjane et al., 2020). For mounting, first a bottom sapphire disk (diameter 6 mm, thickness 120 μm; Leica or Wohlwend, Sennwald, Switzerland) was placed on the middle plate of a clear cartridge. Then the spacer ring (outer diameter 6 mm, inner diameter 5 mm; thickness 200 μm) was put with a drop of ACSF containing 15% of the cryoprotectant polyvinylpyrrolidone over the ring to adhere it to the sapphire. Next, the tissue sample was transferred. Finally, the second sapphire disk was put on top to close the sandwich. Slices were transferred carefully but quickly, using a paintbrush number 4 covered with solution. During the entire mounting procedure, the specimen assembly table and the freezing chamber of the Leica EM ICE were kept at 37°C. To keep track of the light-stimulated side, slices from left and right hemispheres were frozen separately.

**Freeze substitution, embedding, and electron microscopy**

Freeze substitution was performed using an AFS1 or AFS2 freeze substitution system (Leica) equipped with an agitation module, as previously described (Borges-Merjane et al., 2020). For the first day of freeze substitution, the high-pressure frozen samples were transferred from liquid nitrogen to vials with 0.1% tannic acid in acetone, frozen in liquid nitrogen. Vials were then transferred to glass dishes with acetone at room temperature for inspection, to ensure that slices were intact and sandwiches properly opened and infiltrated. Samples were then washed with propylene oxide (2×10 min), and infiltrated with Durcupan resin at 2:1, 1:1, and 1:2 propylene oxide/Durcupan resin mix (1 h at room temperature), shaking. They were then left in pure resin overnight at room temperature, and embedded in BEEM capsules (Electron Microscopy Sciences, Hatfield, PA, USA). Capsules were polymerized overnight at 100°C. Slices were carefully embedded with the light-stimulated side facing down, for ultramicroscopy.

Embedded capsules were trimmed with glass knives and ultrathin sections were taken in a Leica EM UC7 Ultramicrotome with Diatome Histo (for 70-nm sections) diamond knives. Sections were kept in slot grids with formvar for TEM imaging. Post-staining with a mono-exponential function plus an offset. For analysis of PTP magnitude and time course, only pairs with stationary control or 100 stimuli, the peak amplitude of each EPSC was measured directly from the preceding baseline. PTP decay time course was fit with a mono-exponential function plus an offset. For analysis of PTP magnitude and time course, only pairs with stationary control conditions, as indicated by lack of significant correlation in the baseline epoch, were used. PTP was computed as Max[EPSCt after HFS] / Mean[EPSCt in control], where Max is the maximal EPSCt at 20, 40, or 60 s after HFS, and Mean is the mean of 5 EPSCt control values before HFS. The paired-pulse ratio EPSC2 / EPSC1 was computed from an average trace (from 5 individual traces) before HFS and a single trace after HFS.

For analysis of RRP size, P, and refilling rate, cumulative EPSC peak amplitude was plotted against time for 10 and 100 stimuli. For analysis with the Schneggenburger-Meyer-Neher method (SMN; Schneggenburger et al., 1998), the last 3–5 and 30–50 data points,
respectively, were fit by linear regression. Traces in which the extent of depression (average of the last three/thirty EPSCs compared
with peak EPSC) was < 50% (Neher, 2015) were excluded from the analysis (18% of traces total). The size of the RRP was determined
as the intersection of the regression line with the ordinate, P1 was measured as the ratio of the first EPSC amplitude over pool size, and
refilling rate was obtained from the slope of the line. For analysis with the Thanawala-Regehr method (TR; Thanawala and Regehr,
2013), the EPSC amplitude data points were fit with a product of an exponential facilitation and an exponential depression function.
The depression component g(t) was extracted, normalized, and integrated to G(t). Finally, the cumulative EPSC peak amplitude data
were fit by the linear combination a ∗ G(t) + b. The size of the RRP was determined as the intersection of the fit curve with the ordinate,
and P1 was measured as the ratio of the first EPSC amplitude over pool size. For both methods (Schneggenburger et al., 1999; Than-
awala and Regehr, 2013), the RRP estimates represent “pool decrement” rather than absolute pool size; the true pool size will be
larger than the estimate (Neher, 2015). Using the correction previously suggested for a single homogeneous pool (Neher, 2015), and
assuming y10 / y0 = 0.5, and p10 / p0 = 6 (Vyleta and Jonas, 2014, their Figure S5B), the correction factor pool size / pool decrement
would be estimated as 1.09. 50-Hz trains of ten stimuli applied every 20 s evoked stationary responses, suggesting that they were
below the PTP induction threshold (stationary test based on linear regression analysis of baseline periods without stimulation; p > 0.2;
10 pairs).

For detection of mEPSCs, a machine-learning based detection algorithm was used (MOD; X.Z., A. Schlägl, D.V., and P.J., unpub-
lished data). Subsets of the data were manually scored, and the filter coefficients of an optimal Wiener filter were computed so that
they reproduced the manual scoring trace as closely as possible. The detection threshold was set to a value that maximized Cohen’s k
coefficient. In the current dataset, the area under the curve (AUC) in a plot of true positive against false positive rate was 0.96 ±
0.005, demonstrating reliable and efficient detection of synaptic events (Pernía-Andrade et al., 2012).

Electron micrograph analysis
Electron micrographs were analyzed with Fiji open source software. Hippocampal mossy fiber terminals in stratum lucidum of CA3b
and CA3c subregions were identified based on large size, multiple active zones, synaptic contacts on thorny exces-
cences of CA3 pyramidal cells, puncta adherentia on dendritic shafts, high density of small clear vesicles, and presence of large
dense core vesicles (Borges-Merjane et al., 2020; Chicurel and Harris, 1992; Rollenhagen et al., 2007). Active zones were defined
as the presynaptic membrane regions directly opposed to postsynaptic densities (asymmetric contacts), accumulation of synaptic
vesicles, and characteristic widening of the synaptic cleft (Chicurel and Harris, 1992; Rollenhagen et al., 2007). Vesicles that were less
than 5 nm from the active zone membrane were considered docked. For quantification of number of docked vesicles per profile and
vesicle diameters, counts were normalized to 100 nm of active zone length. A minimum of 154 active zones profiles were analyzed per
animal, and at least three animals were examined per condition. All vesicle counting and image analysis was done with the exper-
imenter blind to the experimental condition. Depletion data were taken from a previously published Neuroresource paper; experi-
ments for the two datasets were performed in an interleaved manner (Borges-Merjane et al., 2020).

Modeling of pool dynamics
EPSC amplitudes during trains were modeled using a model with three pools (pool0, pool1, and pool2, intended to represent docked,
primed, and superprimed synaptic vesicles), filled from an upstream pool of infinite size via a refilling process. The model was
described by the following differential equations

\[ \frac{dp_i(t)}{dt} = r_{i0} p_1(t) - r_{i1} p_i(t) + r(t), \]

(Eq. 1)

\[ \frac{dp_1(t)}{dt} = r_{10} p_0(t) - r_{11} p_1(t) + r_1 p_1(t) - f(t) P_{12} p_1(t), \]

(Eq. 2)

\[ \frac{dp_2(t)}{dt} = r_{12} p_1(t) - r_{21} p_2(t) - f(t) P_{12} p_2(t), \]

(Eq. 3)

\[ \frac{ds(t)}{dt} = \theta(t) - s(t) / \tau_s, \]

(Eq. 4)

where p_i represents the number of vesicles in pool i (i = 0, 1, 2), r_j is the transition rate between pool i and pool j \((i, j = 0, 1, 2, i \neq j)\), P_{i j} and P_{12} are the release probabilities of primed and superprimed vesicles \((P_{12} > P_{11})\), r is the total refilling rate (comprising refilling from
an infinite reserve pool and multiple forms of endocytosis from the plasma membrane; Chanaday and Kavalali, 2018), f is a facilitation
factor (constrained according to P_r × f ≤ 1), and \(\theta(t)\) is a function representing a sequence of pulses of 1 ms duration and unitary amplitude.
\(r(t)\) was computed as \(r_1 \times s(t)_{\text{ms}} / (s(t)_{\text{ms}} + s_{\text{ref}})_{\text{ms}} + r_0\), where \(s_{\text{ms}}\) is a power coefficient of refilling, and \(r_0\) and \(r_1\) are basal
and activity-dependent refilling rate, respectively. \(r_0\) was set to 0 before and after stimulation periods. Similarly, \(f(t)\) was computed as
\((f_{\text{max}} - 1) \times s(t)^{mf} / (s(t)^{mf} + s_{\text{half}}^{mf}) + 1\), where \(m_{f}\) is the power coefficient of facilitation. Quantal size was assumed as 50 pA. Equations were solved using NDSolve of Mathematica 12.0.

To obtain the model that provided the best fit to our experimental data, datasets were averaged across experiments. Models were fit to all 7 datasets (10 pulses at 50 Hz for control conditions, 100 pulses at 100 Hz for HFS_{100APs}, and 5 times 10 pulses at 50 Hz for test conditions; Figures S4E and S4F), minimizing the sum of squared differences between model and experimental observations. For the second dataset, weight factors were set to 0.2, to compensate for the large number of data points in this set; otherwise, weight factors were set to 1. The total simulation time was set to 122.2 s, to match the experimental time. The model contained 16 free parameters (Table S2). To achieve convergence, we first used a custom-made random search algorithm, in which each starting value was varied by multiplication with a random number drawn from a normal distribution with mean 1 and standard deviation 0.1. Parameter vectors were updated when a reduction of sum of squared differences occurred. For further optimization, FindMinimum of Mathematica was used, which provides an implementation of Brent’s principal axis method (https://reference.wolfram.com/language/tutorial/UnconstrainedOptimizationMethodsForLocalMinimization.html#188073100). As datasets before, during, and after PTP were fit simultaneously, the model described PTP in terms of the filling state of the vesicle pools as a function of time.

**Statistics and conventions**

Statistical significance was assessed using a two-sided Wilcoxon signed rank test for paired comparisons or a two-sided Mann-Whitney U test for unpaired comparisons at the significance level \(P\) indicated. Multiple comparisons were performed with a Kruskal-Wallis test. Statistical testing was performed with Python 2, MATLAB 2016 or 2018, or Mathematica 12. For electrophysiology data, values and symbols with error bars indicate mean ± standard error of the mean (SEM), unless specified differently. For EM data, values and symbols denote mean ± standard deviation. For graphical representation of statistics, * indicates \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\), and **** \(p < 0.0001\). Membrane potentials are given without correction for liquid junction potentials. In total, data reported in this paper were obtained from 43 GC recordings in vivo, 106 mossy fiber terminal–CA3 pyramidal neuron paired recordings in vitro, and 581 active zone profiles analyzed by EM.