Supplementary Materials and Methods

Hippocampal slice preparation

Experiments involving the use of animals were performed in accordance with guidelines provided by the Animal Protection Committee of Laval University. First, the animals were anesthetized with isoflurane. The brain was extracted and immersed in an oxygenated cutting ACSF solution maintained at 4 °C. The cutting ACSF solution contained (in mM): NaCl 87, NaHCO$_3$ 25, KCl 2.5, NaH$_2$PO$_4$ 1.25, MgCl$_2$ 7, CaCl$_2$ 0.5, glucose 25 and sucrose 75 (pH = 7.4, 330 mOsm). The brain was then dissected according to instructions for optimal preservation of the hippocampal mossy fibers (1). The brain hemispheres were glued on the specimen disk of a Leica VT1000S vibratome and submerged in cutting ACSF solution. Slices (300 µm) were cut and transferred to an oxygenated and heated (32 °C) ACSF solution containing (in mM): NaCl 124, NaHCO$_3$ 25, KCl 2.5, MgCl$_2$ 2.5, CaCl$_2$ 1.2 and glucose 10 (pH = 7.4, 300 mOsm). Slices were left to recover for 30 minutes at 32 °C. Slices were then left at room temperature. Experiments were started one hour after the slicing procedure.

Whole-cell patch-clamp recording

Hippocampal slices were maintained under a nylon mesh in a recording chamber under an upright microscope. Borosillicate glass electrodes had a resistance of 3 – 5 MΩ for CA3 pyramidal cell recordings. After obtaining a stable whole-cell configuration, CA3 pyramidal cells were held in voltage-clamp or in current-clamp. Voltage-clamp recordings were performed at -70 mV. Current-clamp recordings were performed at the resting membrane potential of the CA3 pyramidal cells (-70 ± 5 mV). Minimal stimulation of mossy fibers was performed using an electrode positioned in the stratum lucidum and connected to a constant current stimulus isolator (A360, WPI, Florida, USA). The pipette was gently moved in the stratum lucidum until large, fast and facilitating EPSCs could be recorded. The stimulation intensity was then decreased to achieve conditions in which both failures and successes could be observed. To confirm the mossy fiber identity of the recorded EPSCs or EPSPs, DCG-IV (1 µM) was applied in the end of a subset of experiments. Recordings in which the postsynaptic response was decreased by at least 80% were conserved for further analysis. Electrophysiological data was acquired with Molecular Devices equipment (Axopatch 200B amplifier and Digidata 1322A, or MultiClamp 700B amplifier with Digidata 1440A) and the Clampex suite. The electrophysiological data was low-pass filtered at 2 kHz, digitized at 10 kHz

32   and recorded on a personal computer. For calcium imaging experiments, whole-cell patch-clamp

33   recordings were obtained from granule cells with the solution described above, but lacking EGTA.

34   This patch solution was supplemented with 40 µM of the morphological dye Alexa-594 and 375

35   µM of the low-affinity calcium indicator Fluo-4FF. Granule cells were held in the current-clamp

36   mode at their resting membrane potential. Action potentials were evoked by brief current injections

37   (2 ms, 1 – 1.5 nA) in trains of 10 APs, at either 20 Hz or 100 Hz. Glass electrodes used for whole-

38   cell recordings from granule cells had a resistance between 4 – 7 MΩ.

39

40   *Random-access two-photon calcium imaging*

41   Following diffusion for at least 1 hour of the fluorophores in the granule cell, the axon was tracked

42   to the CA3 region (2, 3). Giant MF boutons were unequivocally identified in the CA3 region based

43   on their morphology imaged with the AlexaFluor-594 fluorescence. 20 sites evenly dispersed on

44   the whole bouton were recorded quasi-simultaneously, yielding an imaging speed of 950 Hz. This

45   recording paradigm allowed a good compromise between signal to noise ratio of the signal and the

46   temporal resolutions, and therefore enabled recording calcium elevations generated by high-

47   frequency firing of APs. The very low-affinity Ca2+ indicator Fluo-4FF proved critical to resolve

48   high-frequency bursts of APs evoked at 100 Hz without indicator saturation. We used a custom

49   built random-access two-photon microscope (2). Transmitted photons passed through a high-

50   numerical aperture oil condenser (NA = 1.4) and were low-pass filtered at 720 nm. Photons were

51   separated by a dichroic mirror (580 nm) to independently collect red and green photons. Photons

52   were then band-pass filtered at 500-560 nm for the green channel and 595-665 nm for the red

53   channel. Both the red and the green photons were collected simultaneously. Collection of photons

54   was performed using a pair of AsGaP photomultiplier tubes (H7422P-40, Hamamatsu) located

55   close to the recording chamber. The laser and the acquisition system were controlled by a Labview

56   custom-made software (4).

57

58   *Analysis of electrophysiological and calcium imaging data*

59   Electrophysiological data were analyzed in Clampfit and in Igor Pro. AP probability was

60   calculated from 20 sweeps. To avoid inducing long-term plasticity, sweeps were evoked every 30

61   seconds. EPSC amplitude was measured from the average trace obtained from 20 sweeps. Calcium

62   elevations recorded in giant MF terminals were exported to Excel database. The ΔG/G ratio was

63 calculated for all trials and trials (50 – 140) were averaged together. The peak Ca^{2+} amplitude for

64 individual calcium transients was determined from baseline to peak. In all figures, symbols show

65 the mean and the error bars indicate the SEM.

66

67 *Non-stationary single compartment model of presynaptic Ca^{2+} dynamics*

68 The model is described by the following system of differential equations:

$$\frac{d[Ca^{2+}]}{dt} = j_{Ca} + k_{off}^I[CaI] - k_{on}^I[Ca^{2+}][I] + \sum_i (k_{off}^{B_i}[CaB_i] - k_{on}^{B_i}[Ca^{2+}][B_i]) - P_{rem}$$

$$\frac{d[I]}{dt} = k_{off}^I[CaI] - k_{on}^I[Ca^{2+}][I]$$

$$\frac{d[B_i]}{dt} = k_{off}^{B_i}[CaB_i] - k_{on}^{B_i}[Ca^{2+}][B_i]$$

69

70 where the square brackets denote concentrations, and the superscript indices of the reaction rate

71 constants denote endogenous Ca^{2+} buffers B_i or the Fluo-4FF indicator I . The AP-dependent

72 Ca^{2+} influx time course j_{Ca} was approximated by the Gaussian function

73 $j_{Ca} = \frac{\Delta[Ca^{2+}]_{total}}{\sigma\sqrt{2\pi}} \sum_i \exp\left(-\frac{(t-t_i^{AP})^2}{2\sigma^2}\right)$, where t_i^{AP} denotes the times of peaks of Ca^{2+} currents

74 during each action potential. The use of the low affinity Ca^{2+} indicator Fluo-4FF ($K_d = $ 9.7 µM)

75 did not allow us to estimate resting $[Ca^{2+}]_{rest}$ reliably, which in turn prevented the numerical

76 estimation of the total volume averaged presynaptic Ca^{2+} entry $\Delta[Ca^{2+}]_{total}$. Therefore we used

77 previous estimates for both $[Ca^{2+}]_{rest} = 75$ nM and $\Delta[Ca^{2+}]_{total} = 50$ µM obtained with high affinity

78 Ca^{2+} indicators (3, 5). Because in our experimental conditions $[Ca^{2+}]_{ext} = $ 1.2 mM (in comparison

79 to $[Ca^{2+}]_{ext} = $ 2 mM in ref. (3)) we reduced $\Delta[Ca^{2+}]_{total}$ determined in ref. (3) by a factor of 1.5

80 based on the dependency of VGCC conductance on $[Ca^{2+}]_{ext}$. Ca^{2+} removal was approximated by

81 a first-order reaction $P_{rem} = k_{rem}([Ca^{2+}] - [Ca^{2+}]_{rest})$. We assumed that a MFB terminal contains

82 three endogenous buffers ATP, CB and CaM. The complete set of model parameters and Ca^{2+}

83 binding reactions, including concentrations and binding properties of the endogenous buffers,

84 which were previously determined using combination of electrophysiological, imaging and

85 immunocytochemical methods (6-9) is specified in **Supplementary Table 1**. The model was

86   numerically solved using the adaptive step-size Runge-Kutta algorithm. The model operated with

87   only two adjustable (free) parameters: the unknown ratio between resting Fluo-4FF fluorescence

88   signal and the background fluorescence and Ca^{2+} removal rate k_{rem}. Both parameters were

89   constrained by a straightforward fitting procedure that would match the calculated and

90   experimental fluorescence profiles.

91

92   *Spatial VCell model of MFB Ca^{2+} dynamics*

93   A simulation unit, representing part of a MFB terminal with a single active zone (AZ), was

94   modeled as a parallelepiped of size $x = 0.5\ \mu m$, $y = 0.5\ \mu m$ and $z = 0.79\ \mu m$. The AZ was

95   located in the XY base ($z = 0.79\ \mu m$) and contained a single rectangular VGCC cluster of

96   dimensions $40\ nm \times 80\ nm$ placed in the center of the AZ. The size of XY base corresponded to

97   the average distance among different AZs in MFB terminals ($0.5\ mm$) (10). The height of the

98   simulation unit was adjusted to $z = 0.79\ mm$ in order to match the magnitude of local VGCC-

99   mediated Ca^{2+} influx at the AZ (see below) to the value of experimentally estimated $\Delta[Ca^{2+}]_{total} =$

100   33.3 µM. We assumed that 28 VGCCs were evenly distributed within the VGCC cluster (11, 12).

101   The average AP-evoked Ca^{2+} current was simulated using the five-state VGCC gating kinetic

102   model in MFB (11) using the NEURON simulation environment (13, 14) and the experimentally

103   determined MFB AP waveform (11), which was considered to be constant during burst of APs.

104   Ca^{2+} extrusion by the bouton surface pumps (excluding the AZ) was approximated by a first-order

105   reaction $j_{extr} = k_{extr}([Ca^{2+}] - [Ca^{2+}]_{rest})$ (15, 16) located at the XY parallelepiped base opposite to

106   the AZ; k_{extr} was calculated using the experimentally constrained single-compartment model

107   average Ca^{2+} removal rate ($k_{rem} = 400\ s^{-1}$) as $k_{extr} = \dfrac{V}{S} k_{rem} = 320\ \mu m\ s^{-1}$ (where V is the volume

108   of the simulation unit and S is the area of the XY base). In the case of 'Mobile CaM' model we

109   assumed $[CaM]_{total} = 150\ \mu M$ as was estimated in **Supplementary Fig. 10A.** In the case of 'CaM

110   dislocation' model we assumed that all CaM molecules were located within a single 10 nm layer

111   of VCell voxels adjacent to the AZ plasma membrane (i.e. at the 0.5 µm x 0.5 µm bottom base of

112   the simulation unit). Concentration of CaM was 3 molecules / 10 nm x 10 nm x 10 nm voxel, as

113   estimated in **Supplementary Fig. 10C**. The details of 'CaM dislocation' model are described in

114   our previous publication (14). Briefly, we assumed that upon binding of two Ca^{2+} ions by the C-

115   lobe a CaM molecule can irreversibly dissociate from the plasma membrane (k_{off}^{CaM} = 650 s^{-1}, ref.

116   (14)) and freely diffuse in the cytosol.

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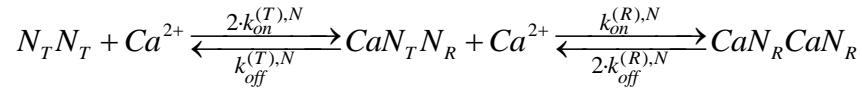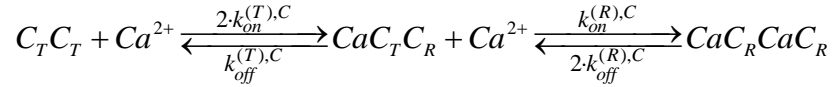133

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137 **Supplementary Table**

138 **Supplementary Table 1.**

139 **Properties of endogenous and exogenous Ca^{2+} buffers used in non-stationary single**

140 **compartment and in multi-compartment VCell models of presynaptic MFB Ca^{2+} dynamics.**

Fluorescent Ca^{2+} indicator Fluo-4FF

Reaction:

$$Fluo4FF + Ca^{2+} \underset{k_{off}^{Fluo4FF}}{\overset{k_{on}^{Fluo4FF}}{\rightleftarrows}} CaFluo4FF$$

Reaction rates:

$k_{on}^{Fluo4FF}$	600 µM^{-1} s^{-1}
$k_{off}^{Fluo4FF}$	5,820 s^{-1}
$[Fluo4FF]_{total}$	375 µM

ATP (Supplementary refs. (7, 9, 14, 16, 17))

Reactions:

$$ATP + Ca^{2+} \underset{k_{off}^{ATP}}{\overset{k_{on}^{ATP}}{\rightleftarrows}} CaATP$$

Reaction rates:

k_{on}^{ATP}	500 µM^{-1} s^{-1}
k_{off}^{ATP}	1.0 x 10^5 s^{-1}
$[ATP]_{total}$	0.9 mM (corresponding to 58 µM $[ATP]_{free}$ at 1 mM $[Mg^{2+}]_{free}$)
D_{ATP}	220 µm^2 s^{-1}

Calbindin-D$_{28K}$ (Supplementary refs. (8, 18))

Reactions:

$$CB_{fast} + Ca^{2+} \underset{k_{off}^{CB\_fast}}{\overset{k_{on}^{CB\_fast}}{\rightleftarrows}} CaCB_{fast}$$

$$CB_{slow} + Ca^{2+} \underset{k_{off}^{CB\_slow}}{\overset{k_{on}^{CB\_slow}}{\rightleftarrows}} CaCB_{slow}$$

Reaction rates:

$k_{on}^{CB\_fast}$	87 µM^{-1} s^{-1}
$k_{off}^{CB\_fast}$	35.8 s^{-1}
$[CB_{fast}]_{total}$	80 µM
$k_{on}^{CB\_slow}$	11 µM^{-1} s^{-1}
$k_{off}^{CB\_slow}$	2.6 s^{-1}
$[CB_{slow}]_{total}$	80 µM
D_{CB}	50 µm^2 s^{-1}

Calmodulin (Supplementary refs. (9, 14))

Reactions:
N-lobe

$$N_T N_T + Ca^{2+} \xleftrightarrow[k_{off}^{(T),N}]{2 \cdot k_{on}^{(T),N}} CaN_T N_R + Ca^{2+} \xleftrightarrow[2 \cdot k_{off}^{(R),N}]{k_{on}^{(R),N}} CaN_R CaN_R$$

C-lobe

$$C_T C_T + Ca^{2+} \xleftrightarrow[k_{off}^{(T),C}]{2 \cdot k_{on}^{(T),C}} CaC_T C_R + Ca^{2+} \xleftrightarrow[2 \cdot k_{off}^{(R),C}]{k_{on}^{(R),C}} CaC_R CaC_R$$

Reaction rates for mobile CaM:
N-lobe

$k_{on}^{(T),N}$	770 $\mu M^{-1} s^{-1}$
$k_{off}^{(T),N}$	1.6 x 10^5 s^{-1}
$k_{on}^{(R),N}$	3.2 x 10^4 $\mu M^{-1} s^{-1}$
$k_{off}^{(R),N}$	2.2 x 10^4 s^{-1}

C-lobe

$k_{on}^{(T),C}$	84 $\mu M^{-1} s^{-1}$
$k_{off}^{(T),C}$	2.6 x 10^3 s^{-1}
$k_{on}^{(R),C}$	25 $\mu M^{-1} s^{-1}$
$k_{off}^{(R),C}$	6.5 s^{-1}
$[CaM]_{total}$	150 μM (mobile), 3 molecules / voxel (dislocation model)
D_{CaM}	50 $\mu m^2 s^{-1}$

In the case of CaM associated with membrane via interaction with neuromodulin we assumed that $k_{off}^{(R),C}$ was increased 50-fold ($k_{off}^{(R),C}$ = 325 s^{-1}) (19, 20). We also assumed that upon Ca^{2+} binding by the C-lobe there was a 50% chance of CaM dissociation from neuromodulin (i.e k_{off}^{CaM} = $2 \times k_{off}^{(R),C}$ = 650 s^{-1}, see ref. (14) for details. $[CaM]_{total}$ was constrained in **Supplementary Fig. 10.**

Fast high-affinity exogenous Ca^{2+} buffer mimicking behavior of endogenous MFB Ca^{2+} buffers (Supplementary ref. (12))
Reaction:

$$B + Ca^{2+} \xleftrightarrow[k_{off}^{B}]{k_{on}^{B}} CaB$$
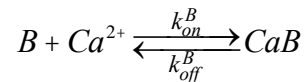
Reaction rates:

k_{on}^{B}	800 $\mu M^{-1} s^{-1}$
k_{off}^{B}	176 s^{-1}
$[B]_{total}$	0.3 mM
D_B	50 $\mu m^2 s^{-1}$

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Supplementary Figure Legends

Supplementary Figure 1 – MFBs count APs at any frequency and number

(**A**) Representative voltage-clamp recordings of MFB evoked EPSCs in a CA3 cell. To explore whether AP counting occurs for any number of APs (in a physiological range), the number of stimuli in trains evoked at 20 Hz was varied between 1 and 10. The last stimulus was kept constant at 100 Hz. The amplitude of the last EPSC was measured. Gray traces show individual trials. Black traces show the average of 20 trials. (**B**) Normalized EPSC amplitude as a function of stimulus number for experiments illustrated in (A) (n = 25 for 100 Hz; n = 7 for each experiment with variable number of stimuli). Short-term facilitation was virtually identical for combined 20 Hz and 100 Hz trains of stimuli described in (A), when compared to trains evoked purely at 100 Hz. (**C**) Trains consisting of 5 stimuli at 10, 20 or 100 Hz were followed by a single stimulus at a constant 100 Hz to investigate if AP counting occurs at any frequency (in the physiological range of granule cell firing). Plotting the amplitude of the 6th EPSC as a function of prepulse frequency shows that the size of the 6th EPSC is independent of the prepulse frequency. Gray traces show individual neurons. The black trace is the average of n = 10 cells.

Supplementary Figure 2 – Post-tetanic potentiation changes the number of APs required to trigger CA3 pyramidal cell firing

(**A**) Example traces of current-clamp recordings before and after the induction of PTP. Red arrows point to the first AP evoked by the stimulation trains. Insets show the expanded traces, focusing on the first 3 EPSPs. Note the truncated AP in the trace recorded following PTP. (**B**) Amplitude of the first EPSP in time. PTP was induced at t = 200 s by a single train of 100 stimuli evoked at 100 Hz (n = 6). (**C**) Position of the first observed AP during the train as a function of time. In control condition, the first EPSP was generally observed at the 6th stimulus. PTP decreased the number of stimuli required to trigger CA3 pyramidal cell firing to 3, a value which gradually recovered to control value within 100 s (n = 6).

Supplementary Figure 3 – The rate of asynchronous release depends on the stimulation frequency

(**A**) Voltage-clamp recordings showing the expanded portion of traces following 10 stimuli delivered at either 20 Hz (top) or at 100 Hz (bottom). Recordings shown are the overlay of 20 consecutive traces. (**B**) Rate of asynchronous release as a function of stimulation frequency (20 Hz: n = 6; 50 Hz: n = 8; 100 Hz: n = 8; 200 Hz: n = 3). For 100 Hz stimulation, the average frequency of asynchronous events was 5 Hz. This corresponds to ~ 0.05 quantal events during 10 ms inter-spike interval. Considering that the average quantal size in our recordings was 50 pA, the asynchronous release will contribute only ~ 2.5 pA over a 10 ms window. On the other hand the amplitude of synchronous EPSCs recorded during 6AP trains was two orders of magnitude higher, ranging from ~ 125 pA (1st AP) to ~ 550 pA (6th AP).

Supplementary Figure 4 – Amplitudes of individual AP-evoked presynaptic Ca^{2+}-fluorescence transients are independent of spike number and burst frequency.

Left, Representative image of a MFB labelled with AlexaFluor-594 with 20 recording sites for RAMP imaging shown with orange circles. An associated filopodia is visible to the right of the bouton. Middle, Corresponding Fluo-4FF experimental traces (black) in response to a 5X20 Hz + 1X100 Hz AP burst (average of 181 sweeps). Red trace shows the non-stationary model fit. Right, Normalized peak amplitude of AP-evoked Fluo-4FF fluorescence as a function of AP number. Note that the amplitude of Ca^{2+} fluorescence elevations remains constant during trains with variable frequency (n = 3).

Supplementary Figure 5 – A single fast high-affinity buffer fails to replicate the experimental Ca^{2+} imaging data.

(**A,B**) Single compartment model-computed fluorescence traces during 10X20 Hz (**A**) and 10X100 Hz (**B**) AP stimulation for the case of endogenous buffers (CB, CaM and ATP) or for the case of a single fast high-affinity exogenous buffer (Supplementary Table 1), which was shown to mimic the effect of the endogenous buffers on vesicular release in MFB terminals (12). (**C,D**) Normalized peak calcium transient amplitude as a function of AP number for experimental Fluo-4FF data (black trace), a model computed traces for 10X20 Hz (**C**) and 10X100 Hz (**D**) stimulation trains (n = 7 boutons for experimental data).

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Supplementary Figure 6 – Monte Carlo realization of AP-evoked response

(**A**) Three representative Monte Carlo realizations of computed EPSCs in response to 6X100 Hz AP train in a MFB. Each trace is a sum of 125 individual Monte Carlo runs, corresponding to readily releasable pool size (RRP) m = 125. Insert, quantal response (this was used to compute EPSCs). (**B**) Individual realisations (gray traces, as shown in (A)) and average from 60 000 individual realisations (black trace).

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Supplementary Figure 7 – Constraining the vesicle replenishment rate

(**A**) Representative recording of CA3 pyramidal cell EPSCs during a 50X100 Hz train of stimuli used to evaluate the vesicle re-priming rate. (**B**) Responses to the first 10 stimuli and last 10 stimuli on (a) are shown enlarged. Light traces are individual trials. Black trace is the average of 7 trials. (**C**) Cumulative EPSC amplitude as a function of stimulus number. Modelling with various replenishment rates are shown in color. The experimental data (average of n = 9 cells) was best fitted with a replenishment time τ = 50 ms, corresponding to the replenishment rate of $k_{rep} = 20$ s^{-1}.

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Supplementary Figure 8 – Simulated MFB Ca^{2+} dynamics and EPSCs during 5X100 + 1X20 Hz and 6X20 Hz stimulation

Comparison of $[Ca^{2+}]$ dynamics during 5X100 Hz + 1X20 Hz stimulation (**A**) and 6X20 Hz stimulation (**B**). (**C**) [Ca^{2+}]$_{residual}$ and $[Ca^{2+}]_{amplidude}$ during the trains of APs shown in (A) and (B). Simulated EPSCs for 5X100 Hz + 1X20 Hz stimulation (**D**) and 6X20 Hz stimulation (**E**). Average of M = 60,000 Monte Carlo runs for each paradigm scaled for RRP of size m = 125. (**F**) EPSC amplitude as a function of AP number for the simulations shown in (D) and (E).

226

Supplementary Figure 9 – 'Mobile CaM' model, endogenous buffer dynamics during stimulation trains

229 (**A-C**) Endogenous buffers dynamics during 5X20 Hz + 1X100 Hz (red) and 6X100 Hz (green).

230 (**D-F**) Endogenous buffers dynamics during 5X100 Hz+ 1X20 Hz (blue) and 6X20 Hz (black) AP

231 trains.

232

233 **Supplementary Figure 10 – Estimating CaM concentration in MFB terminals**

234 (**A**) The precise [CaM]$_{total}$ in MFB is unknown. To estimate this value we computed the average

235 release probability of individual readily releasable vesicles (p_v) in response to a single AP for

236 different [CaM]$_{total}$ for the case of 'Mobile CaM' model. A previous study used dialysis of MFB

237 terminals with fixed concentrations of BAPTA to demonstrate that the effect of MFB endogenous

238 buffers on vesicular release can be approximated by a single fast high-affinity Ca^{2+} exogenous

239 buffer (12). Therefore, as a reference point we used p_v value computed in our modelling

240 framework for the case of such fast high-affinity buffer (see Supplementary Table 1). As a result

241 we obtained an estimate for [CaM]$_{total}$ = 150 µM which was within experimentally estimated range

242 of neuronal [CaM]$_{total}$ (9). (**B**) The steep dependency of p_v on [CaM]$_{total}$ suggested a dominant

243 effect of CaM N-lobe on $[Ca^{2+}]$ transient at the release site. In agreement with this removal of CB

244 or CaM C-lobe from the model resulted in only minor increase in AP-evoked $[Ca^{2+}]_{peak}$ and

245 simulated EPSC amplitude. In contrast removal of CaM N-lobe resulted in ~2.7 fold increase of

246 $[Ca^{2+}]_{peak}$ and ~9.5 fold increase of EPSC amplitude. (**C**) Constraining [CaM]$_{total}$ in the AZ in the

247 limiting case of 'CaM dislocation' model. In this case we considered that at the beginning of VCell

248 simulations all CaM molecules were located within a 10 nm layer of voxels located at the bottom

249 plane of the modelling unit corresponding to the AZ (**Fig. 4 A,F**). Vesicular release probability p_v

250 was calculated for different number of CaM molecules in a 10 nm x 10 nm x 10 nm voxel and

251 compared to the same reference point as in (A). As a result we obtained an estimate for the local

252 [CaM] in the AZ ~ 3 molecules / voxel.

253

254 **Supplementary Figure 11 – Effect of CaM dislocation on active zone Ca^{2+} dynamics and**

255 **vesicular release**

(**A**) Snapshots of spatial distribution of normalized total [CaM] (which accounts for all CaM molecules irrespective of their Ca^{2+} binding state) in the AZ plane, illustrating progressive dislocation of CaM from the membrane during AP stimulation predicted by 'CaM dislocation model' for 5X100 Hz + 1X20 Hz and 6X20 Hz stimulation patterns. (**B**) VCell-computed $[Ca^{2+}]$ transients at the release site during 5X100 Hz + 1X20 Hz and 6X20 Hz AP trains and (**C**) corresponding simulated EPSCs for the case of 'CaM dislocation' model. (**D**) Summary graph showing that experimentally observed short-term facilitation levels are likely to be explained by joint contribution of the two limiting cases represented by 'Mobile CaM' (low facilitation) and by 'CaM dislocation' (high facilitation) models that both allow AP counting logic.

Supplementary Figure 12 – Vesicle occupancy of the release site during AP stimulation

Vesicle occupancy as a function of time for 5X20 Hz + 1X100 Hz (red) and 6X100 Hz (green) and 5X100 + 1X20 Hz (blue) and 6X20 Hz (black) for both the 'Mobile CaM' and 'CaM dislocation' models. The vesicle occupancy is higher for lower stimulation frequency due to vesicle re-priming.

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340

Supplementary Figure 1

A

Control

Post-PTP

Post-PTP

Ctl

130 s after PTP

50 ms

20 mV

B

C

100 Hz (1 s)

Supplementary Figure 2

A

10 X 20 Hz

10 X 100 Hz

200 pA

20 ms

B

Supplementary Figure 3

5X20 Hz + 1X100 Hz

$k_{rem} = 0.25$ ms^{-1}

0.1 F/F$_0$

20 ms

40 mV

Fluo4FF: 5X20Hz + 1X100Hz

Norm. Individual Peak Amplitude

AP Number

Supplementary Figure 4

A

10X20 Hz

CB, CaM and ATP   Single fast high-affinity exogenous buffer

100 ms

B

10X100 Hz

CB, CaM and ATP   Single fast high-affinity exogenous buffer

50 ms

C

- CB, CaM and ATP
- Single fast high-affinity buffer
- Experimental data (Fluo4FF)

D

- CB, CaM and ATP
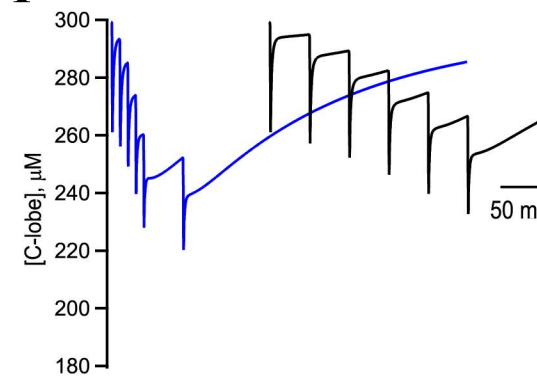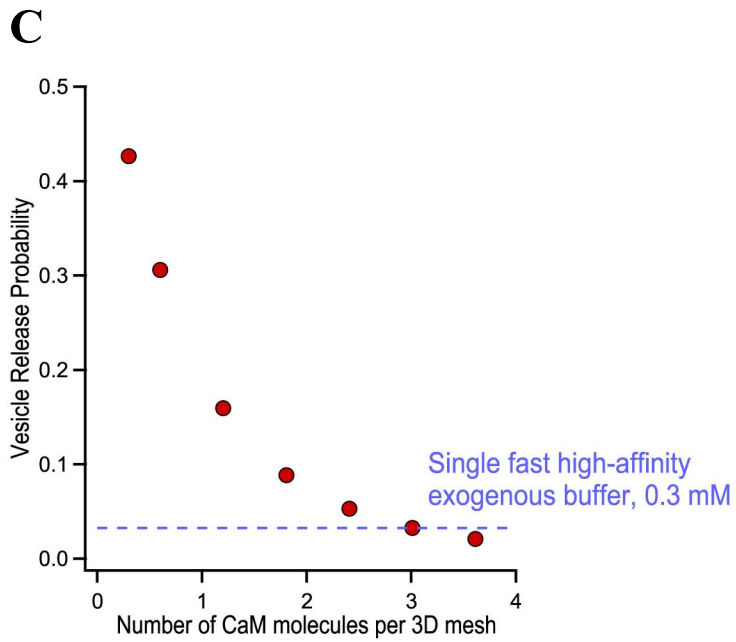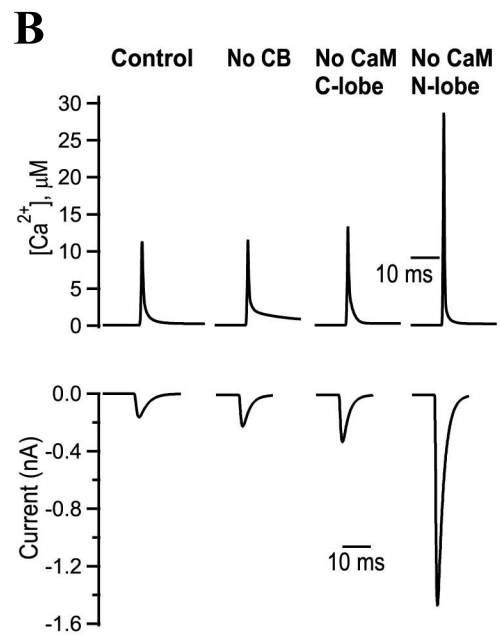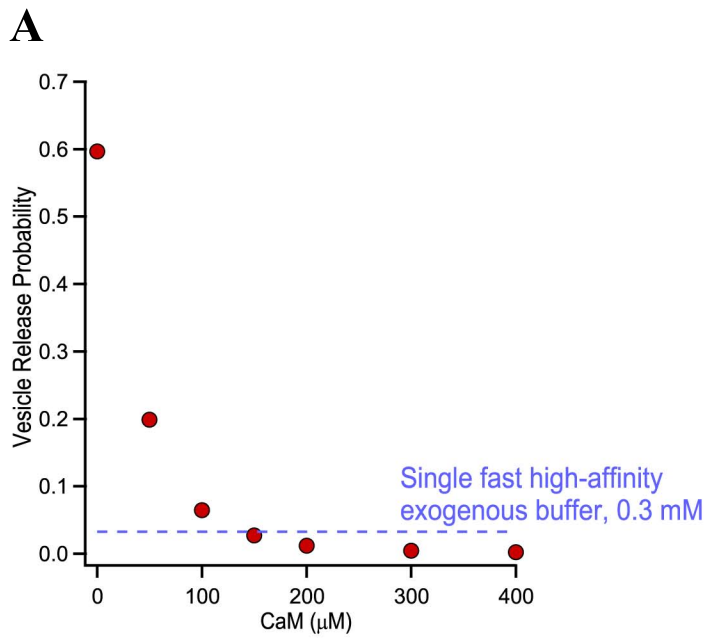- Single fast high-affinity buffer
- Experimental data (Fluo4FF)

Supplementary Figure 5

Supplementary Figure 6

A

B First 10 stimuli

Last 10 stimuli

300 pA

10 ms

C

○ Experimental data

Cumulative EPSC Amplitude (nA)

Number of stimuli

Instant replenishment

10 ms

25 ms

50 ms

75 ms

150 ms

200 ms

No replenishment

300 pA

50 ms

Supplementary Figure 7

Supplementary Figure 8

A 5X20 Hz + 1X100 Hz and 6X100 Hz
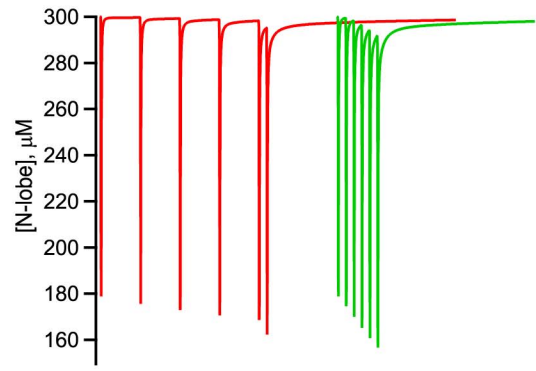
B
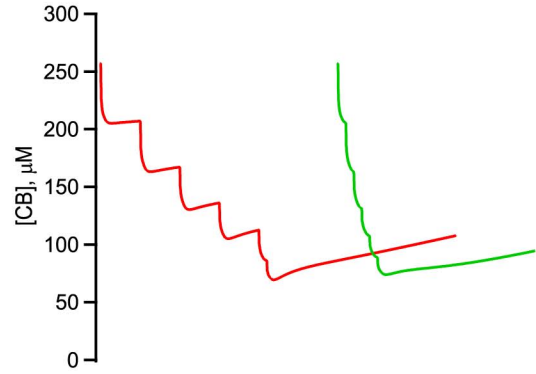
C
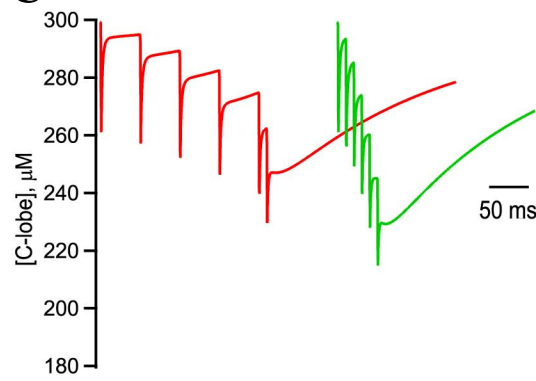
D 5X100 Hz + 1X20 Hz and 6X20 Hz

E

F

Supplementary Figure 9

A

B

C

Supplementary Figure 10

A 5X100 Hz + 1X20 Hz

| 0 ms | 10 ms | 20 ms | 30 ms | 40 ms | 90 ms |

Before 1ˢᵗ AP   Before 2ⁿᵈ AP   Before 3ʳᵈ AP   Before 4ᵗʰ AP   Before 5ᵗʰ AP   Before 6ᵗʰ AP

6X20 Hz

| 0 ms | 50 ms | 100 ms | 150 ms | 200 ms | 250 ms |

Before 1ˢᵗ AP   Before 2ⁿᵈ AP   Before 3ʳᵈ AP   Before 4ᵗʰ AP   Before 5ᵗʰ AP   Before 6ᵗʰ AP

Norm. [CaM] in AZ

B

C 5X100 Hz + 1X20 Hz

6X20 Hz

200 pA

20 ms

D
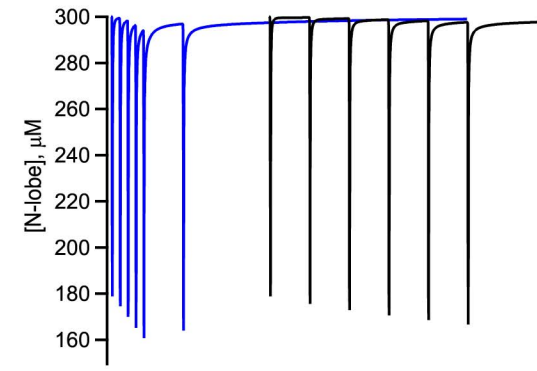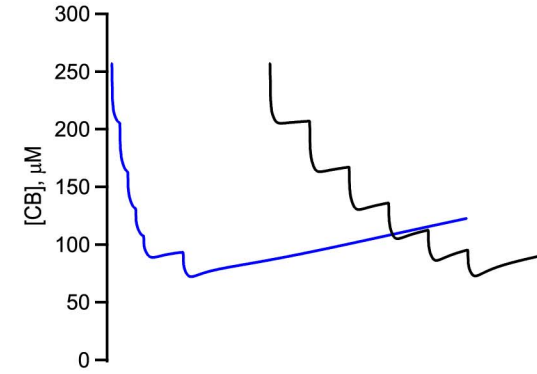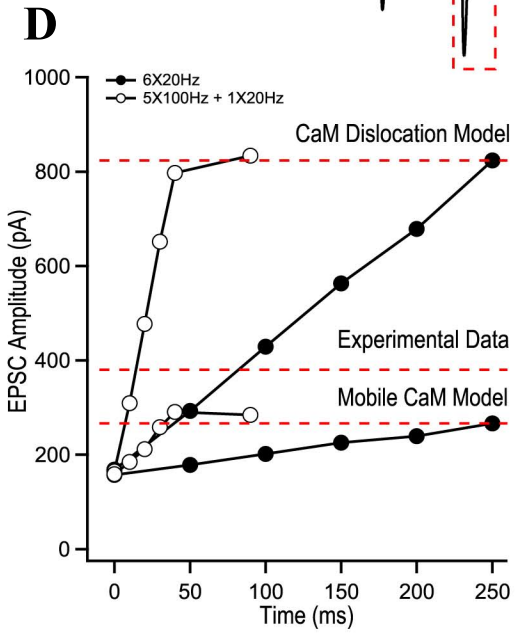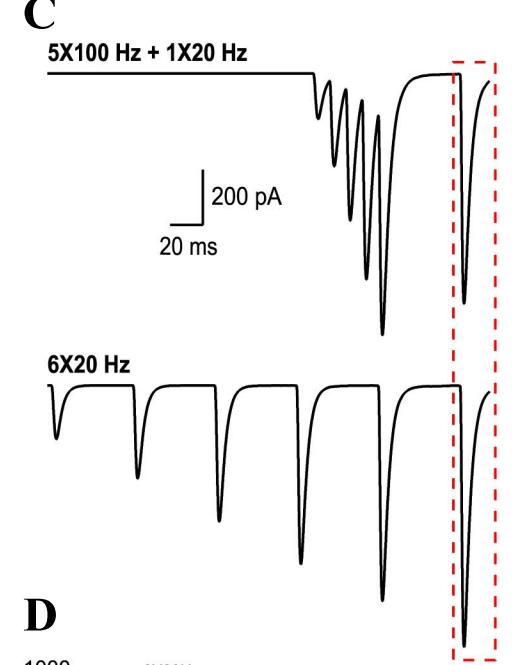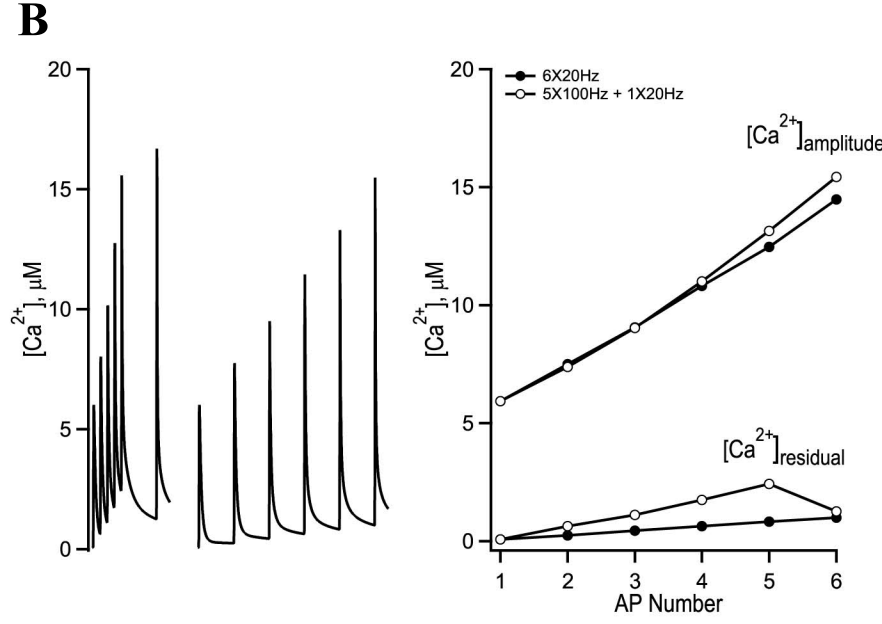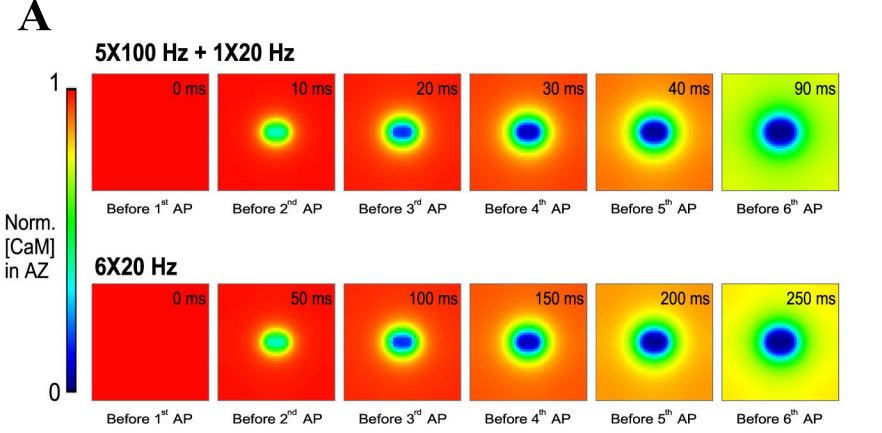
Supplementary Figure 11

Mobile CaM

5X20 Hz + 1X100 Hz
6X100 Hz

5X100 Hz + 1X20 Hz
6X20 Hz

50 ms

CaM Dislocation

50 ms

50 ms

Occupancy

Supplementary Figure 12