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Supplemental Information

Single synapse glutamate imaging reveals multiple levels of release mode regulation in mammalian synapses

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Supplementary Information

Transparent Methods

Constructs

pAAV.hSyn.iGluSnFr.WPRE.SV40 and pCMV(MinDis).iGluSnFR were a gift from Loren Looger (Addgene plasmid # 41732 ; http://n2t.net/addgene:41732 ; RRID:Addgene_41732, and Addgene viral prep # 98929-AAV1; http://n2t.net/addgene:98929 ; RRID:Addgene_98929, respectively) (Marvin, Borghuis et al. 2013). pAAV.hSynapsin.SF-iGluSnFR.A184S was a gift from Loren Looger (Addgene viral prep # 106174-AAV1 ; http://n2t.net/addgene:106174 ; RRID:Addgene_106174).

Neuronal cultures

Primary hippocampal cultures were prepared from postnatal day 0 (P0) rat brains as described previously (Kruger, Favaro et al. 2013), and immobilized on poly-D-Lysine (PDL)-coated glass coverslips. For dendritic measurements, neurons were infected at 2-4 day *in vitro* (DIV) with an adeno-virus associated construct (pAAV.hSyn.iGluSnFr.WPRE.SV40 (Marvin, Borghuis et al. 2013) to express the sensor at the surface of the neurons under human synapsin-1 promoter. The infected cells were then recorded at 17-21 DIV.

For axonal measurements, sparse expression of iGluSnFR in cultured neurons was achieved by transfection of pCMV(MinDis). iGluSnFR construct (Marvin, Borghuis et al. 2013) at 4-7 DIV using Lipofectamine, 2000 (Invitrogen) following the manufacturer's instructions. Imaging was performed on a labeled cell whose neighboring cells were not fluorescent.

For SF-iGluSnFR measurements, neurons were infected at 2-4 DIV with pAAV.hSynapsin.SF-iGluSnFR.A184S, and imaged at 17-21 DIV.

Image acquisition

All the experiments were performed at room temperature in Tyrode's buffer (120 mM NaCl, 2.5mM KCl, 10 mM glucose, 10 mM HEPES, pH 7.4, osmolality was adjusted to that of the culture medium with sucrose). To perform action potential-evoked glutamate release, we used a custom-made imaging chamber with two electrodes for electrical stimulation. The imaging chamber was mounted on the stage of a Nikon Eclipse Ti inverted microscope equipped with a PFS focus controller, a prime 95B scientific CMOS (Photometrics) camera and a pulse generator (HSE-HA, Harvard Apparatus). Illumination was provided in epifluorescence mode by a Lumen 200 Fluorescence Illumination System (Prior Scientific) using a 485/20-nm bandpass filter and emission was collected through a

60x, 1.49 NA Nikon Apochromat objective using a quadband (405/488/561/647nm, Chroma 89902) emission filter. Optical recording of evoked glutamate release was performed by continuous imaging at 20 Hz after application of 20 field stimuli at 0.5 Hz in the presence of 50 µM AP5 (l-2-amino-5-phosphonovaleric acid) and 10 µM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), to block recurrent network activity. The stimuli were triggered by computer-controlled TTL output and their timing was programmed in VisiView software (Visitron Systems). To be able to sample synapses with different evoked release probabilities, imaging solution was supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$. Spontaneous events were then captured by 5-min continuous imaging at 20 Hz at 4mM $CaCl_2$, in the presence of 0.5 µM tetrodotoxin (TTX) to block action potential firing.

Immuno-labeling

To perform on-stage immune-labeling, we fixed cultured neurons for 10 min with 4% paraformaldehyde immediately after functional imaging. Blocking and permeabilization was performed by 30-min incubation in 2.5% (w/v) bovine serum albumin (BSA)-containing phosphate saline buffer (PBS) supplemented with 10% Normal goat serum and 0.1% (v/v) Triton X-100. This was followed by 30-min incubation in 5% BSA in PBS containing 0.1% (v/v) Triton X-100 and primary antibodies, three times 10-min washing in PBS, 30-min incubation in 5% BSA in PBS containing 0.1% (v/v) Triton X-100 and secondary antibodies, and three times 10-min washing in PBS. Appropriate excitation and emission bandpass filters were used for imaging the labeled neurons.

Image analysis

Time-lapse images were loaded in MATLAB (Mathworks, Natick, MA) and after de-noising and high pass temporal filtering, active synapses were detected as local maxima on the first derivative over time of the image stack. Regions of interest (ROIs) from which fluorescence was to be sampled were defined by dilating the maxima to a disk of radius 4 pixels (700 nm). Taking the first derivative over time allowed us to more clearly resolve fluorescence changes associated with evoked or spontaneous release and localize release sites. The differential filter also corrected slow fluctuations and drift in raw fluorescence allowing for automated threshold based event detection over minutes long image acquisitions.

Release probability calculation

To characterize the release properties of individual synapses, filtered fluorescence traces were used for peak detection. Peaks with the amplitude seven times greater than standard deviation of baseline trace during spontaneous imaging were counted as a successful glutamate release event (Fig. S2). Spontaneous frequency was calculated from the total number of events detected during 5-min

acquisition. Evoked probability for each synapse was obtained by dividing the number of events happening within one frame after stimulation by the total number of stimulation.

Quantal release analysis

To measure the quantal amplitude of evoked glutamate release, we performed 5-min continuous evoked imaging at 20 Hz while applying field stimuli at 0.5 Hz. Active ROIs were detected as described above. Florescence traces of individual ROIs were extracted from the raw image. Next, $\Delta F/F_0$ at all stimuli loci were calculated as the difference between the averaged value of 10 to 4 frames preceding and the three frames spanning the stimuli, divided by background subtracted baseline. Normalized evoke-release amplitudes ($\Delta F/F_0$) were then fit to an Expectation-Maximization (EM) Gaussian Mixture Model (McLachlan 2000). Maximum likelihood estimation was used to determine the best fit for the model for a given number of components. Because the likelihood of a fit increases with the number of free parameters, the quality of models with different numbers of fitted components was evaluated using Akaike information criterion (AIC) (Akaike 1973) in order to balance the goodness of fit with the model complexity. Models with a minimal AIC were selected as the preferred gaussian mixture model.

Sub-pixel localization analysis

Localization analysis was performed by custom-written scripts in MATLAB and Fiji. Briefly, in order to characterize the spatial organization of spontaneous and evoked glutamate release events in a single synapse, we chose synapses with at least 10 evoked responses and at least 5 spontaneous release events (182 synapses in total). The acquired signal of all recorded release events were baseline-subtracted and to estimate the molecular position of the fluorescent emitters their recorded point-spread functions were fitted a 2D Gaussian distribution by a least squares fitting (based on Levenberg-Marquardt algorithm) (Ovesny, Krizek et al. 2014). The resulting list of sub-pixel coordinates ("localizations") were loosely filtered for localization uncertainty (< 150 nm) and standard deviation (σ < 2.5 µm) to remove outliers.

Equal sample sizes of randomly picked localizations from evoked or spontaneous emission events were selected and the distances between all possible neighbors were calculated. The farthest distances from ten different subsets of the same synapse were averaged. These farthest values for each transmission mode of 182 synapses were plotted in a cumulative histogram. The density of spontaneous events occurring in evoked release areas and inside spontaneous release areas were calculated as the number of events divided by the respective areas for each synapse, measured as the 2D convex hull of their respective sub-pixel coordinates.

Spatial correlation analysis

The distances between all pairs of synapses in a given measurement were computed. Pairs of synapses were grouped by their mutual distance between 0 to 45 µm into 2.2 µm bins. For each bin of synapse pairs, the Pearson Correlation Coefficient (PCC) of evoked probability, spontaneous frequency, or flat-field corrected Bassoon intensities were computed. PCC was then plotted as a function of distance and fitted with an exponential to determine the correlation length constant. As shown in Fig. S5A-B, no correlation was observed for randomly generated dataset (blue circles).

Determination of relative protein abundance at single synapses

Image stacks of functional recording together with antibody-labeled images were loaded in MATLAB, and after drift correction, the same analysis as described above was used for ROI detection. The center of ROIs were then superimposed on antibody-labeled images and the integrated intensities were collected form flat-field corrected antibody-labeled images. We then obtained the relative abundance of all the proteins by normalizing the integrated intensity of the protein of interest by the integrated intensity of Bassoon at the same ROI. ROIs with relative protein abundance greater than 1.5 × interquartile range above the third quartile were defined as outliers and removed from further analysis.

To obtain the relative enrichment of tested proteins in spontaneous synapses, the relative abundance of proteins in spontaneous synapses was divided by their corresponding relative abundance in evoking synapses of the same cell.

Normalization of Bassoon intensities

To pool the data from all cells, the flat-field corrected Bassoon intensities of synapses in each cell were normalized to that of synapses of the cell with low evoked probabilities (< 0.1) and low spontaneous frequencies (< 0.005). The normalized Bassoon intensities together with the evoked probabilities and spontaneous frequencies of synapses were compiled from all the cells. To plot the functional properties of the synapses versus their size, the data were binned based on normalized Bassoon intensities and the averaged evoked probabilities and spontaneous frequencies per bin were calculated.

Statistical analysis

Differences between the farthest distance distributions for evoked and spontaneous release were evaluated with the Kolmogorov-Smirnov test (n = 182 synapses).

To cancel out the variation in immune-labeling efficiency between replicates, direct comparisons between the relative abundance of proteins in spontaneous and evoking synapses of the same cell were performed. Statistical significance of differences was evaluated using 2-sided Student's *t*-tests for paired samples.

Pearson's χ^2 test was used to compare the distribution of the number of captured spontaneous events over 5 minutes with the expected number of events assuming a Poisson distribution.

Supplementary Table S1. Antibodies used for immuno-labeling, related to Figure 5.

Antibody	RRID	#Catalog	Company
Bassoon	AB_2290619	141 004	Synaptic Systems
Vti1a	AB_2619865	165 003	Synaptic Systems
VAMP7	AB_2212953	232 003	Synaptic Systems
Syntaxin 12/13	AB_2198238	110 131	Synaptic Systems
VAMP4	AB_887816	136 002	Synaptic Systems
VAMP2	AB_887811	104 211	Synaptic Systems
Synapsin 1	AB_11042000	106 103	Synaptic Systems
Synaptotagmin7	AB_887838	105 173	Synaptic Systems
Doc2b	AB_2619874	174 103	Synaptic Systems
Synaptotagmin1	AB_887832	105 011	Synaptic Systems
Munc18-1	AB_2196686	116 011	Synaptic Systems
Complexin 1/2	AB_2619793	122 003	Synaptic Systems
Munc13-1	AB_887733	126103	Synaptic Systems
CF-680 anti-rabbit IgG H+L		SAB4600200	Sigma-Aldrich Chemie GmbH
Alexa Fluor 488 anti-rabbit IgG H+L	AB_2338052	111 545 144	Jackson ImmunoResearch
Cy5 anti-mouse IgG H+L	AB_2338714	115 175 166	Jackson ImmunoResearch

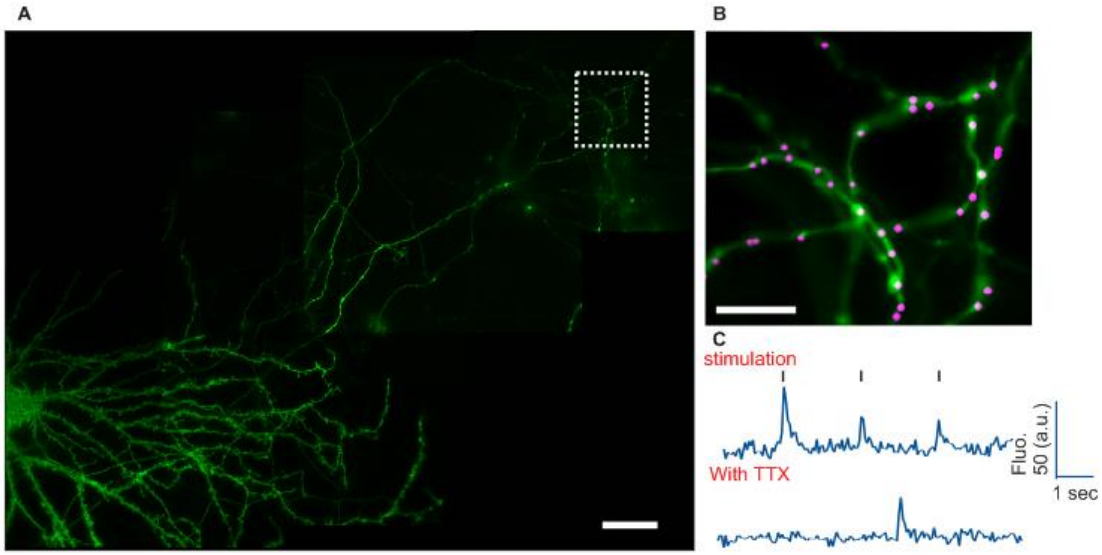

Fig. S1. Detection of glutamate release at axonal terminals, related to Figure 1. (**A**) Sparse expression of iGluSnFR was achieved by chemical transfection of cultured neurons at 4-7 DIV. Evoked and spontaneous glutamate release imaging was performed at the axonal projections of an iGluSnFR-expressing cell. (**B**) Magenta spots represent areas where glutamate release activities were detected. (**C**) Examples of raw fluorescence traces from individual presynaptic boutons upon 0.5 Hz stimulation (top) and during TTX incubation. Scale bars are 40 μm in (**A**) and 10 μm in (**B**).

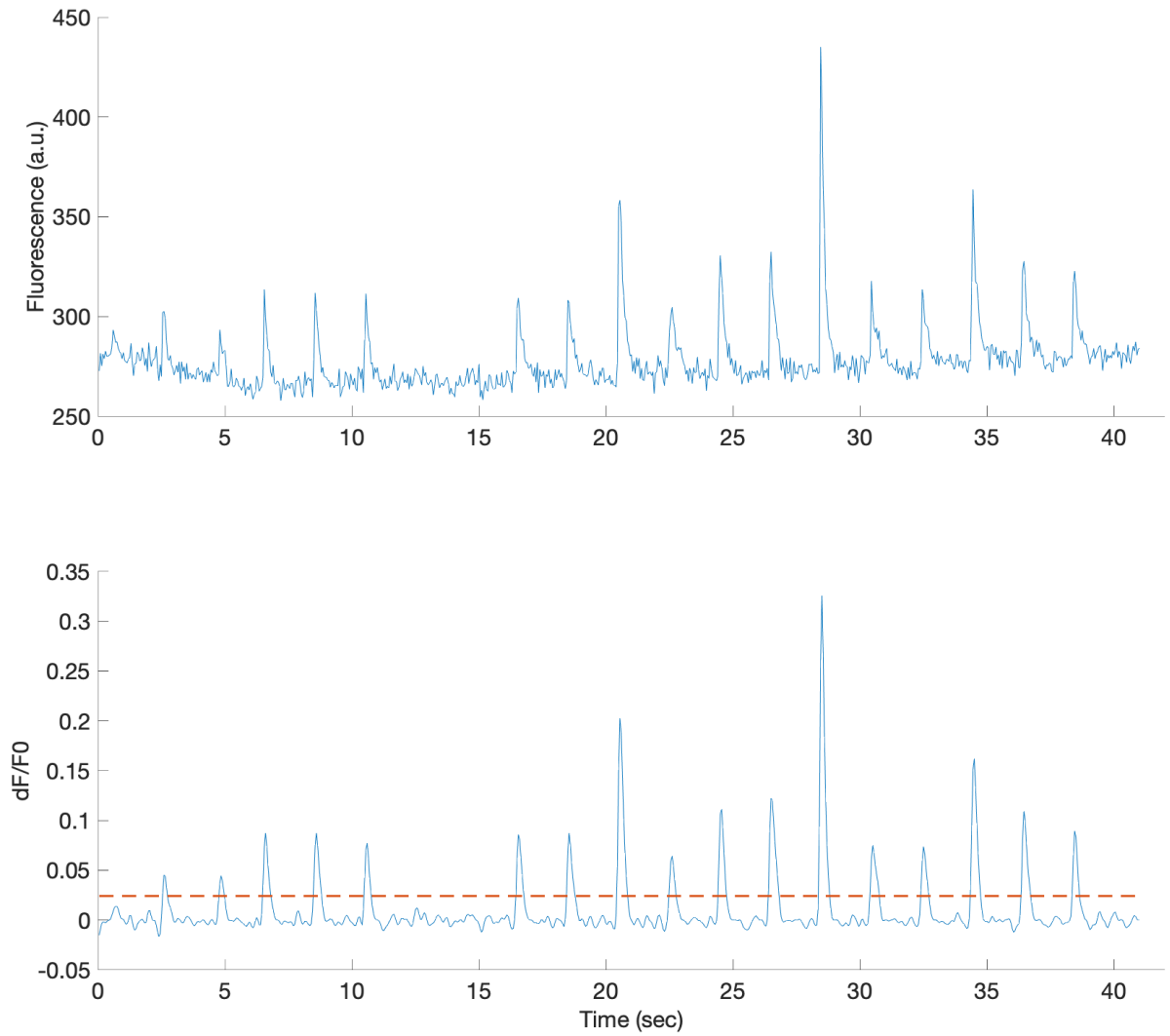

Fig. S2. Detection of successful glutamate release events in individual fluorescence traces, related to Figure 1. Representative raw fluorescence trace (upper graph) as well as its first derivative (bottom graph) recorded from a single synapse in response to 20 electrical stimuli. Dashed line in the bottom graph represent the threshold which was used to define successful release events. Peaks with amplitudes greater than this threshold were counted as a successful glutamate release event

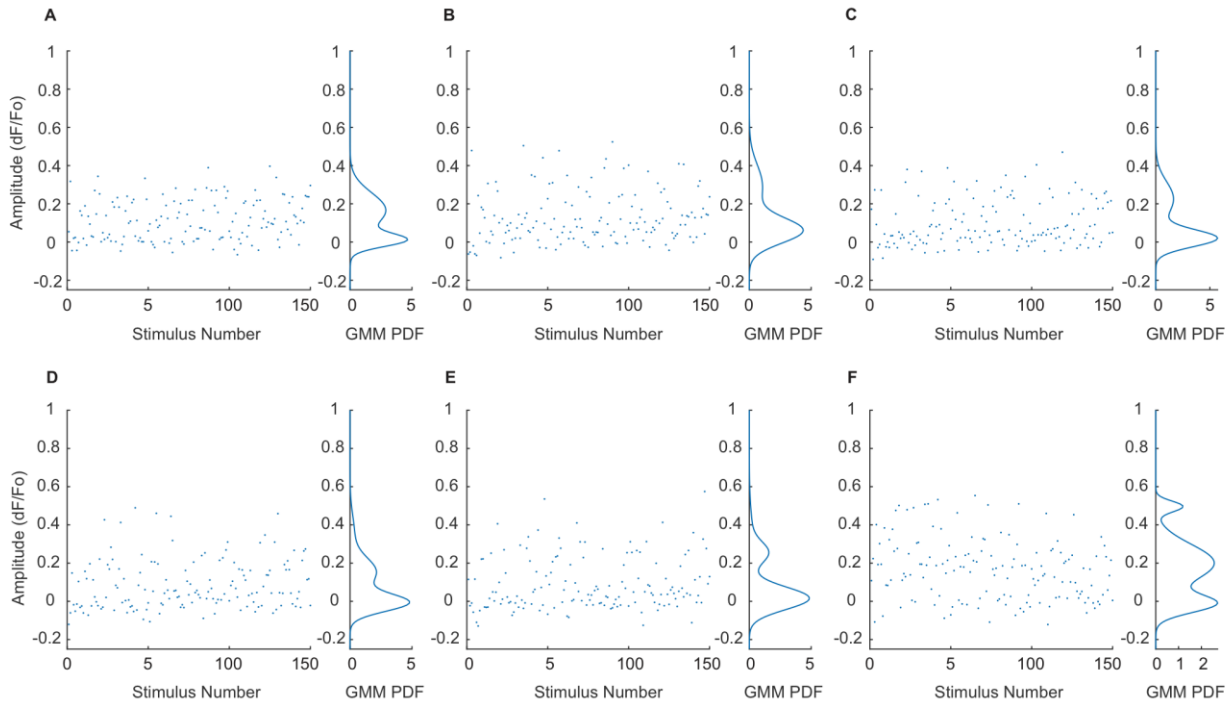

Fig. S3. Quantal analysis of glutamate release at single synapses, related to Figure 2. Fluorescence change magnitudes associated with electrical stimulations were fit for individual synapses with an Expectation Maximization Gaussian Mixture Model. The peaks around zero represent release failures, and the subsequent peaks represent the unitary (A-C) and multi-quantal release (D-F).

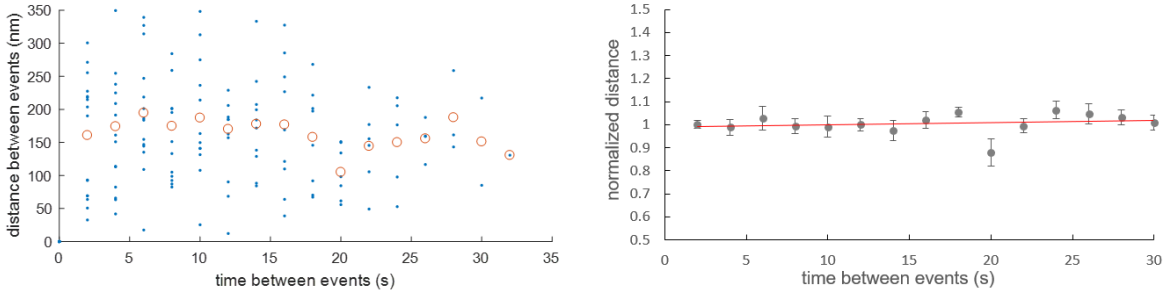

Fig. S4. The distance between release event localizations does not depend on the inter-release interval, related to Figure 3. A) The blue points represent distance between all pairs of release events in a single synapse during 20 AP stimulus train at 0.5 Hz plotted as a function of the time interval between the pair of events. The open red circles represent the mean distance between all pairs of events for a given separation time interval. B) The average of mean normalized localization distance for pairs of release events from 6 synapses are plotted as a function of the time between release events. The mean distance shows no apparent correlation with inter-release interval (Pearson correlation coefficient = 0.20) and a linear fit to the data shows negligible slope (slope = 0.001, R^2 = 0.041).

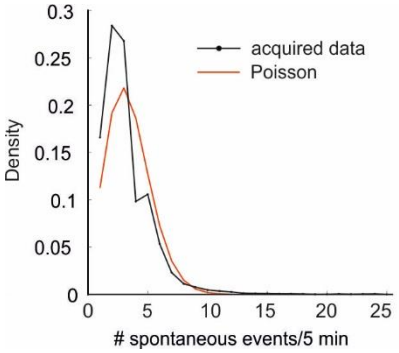

Fig. S5. Non-Poisson distribution of spontaneous events, related to Figure 4. The number of captured spontaneous events over 5-min image acquisition was pooled from more than 24,000 synapses and binned into a histogram (black distribution). The histogram does not fit well with a single Poisson distribution (red fit) ($p < 0.00001$, χ^2 test).

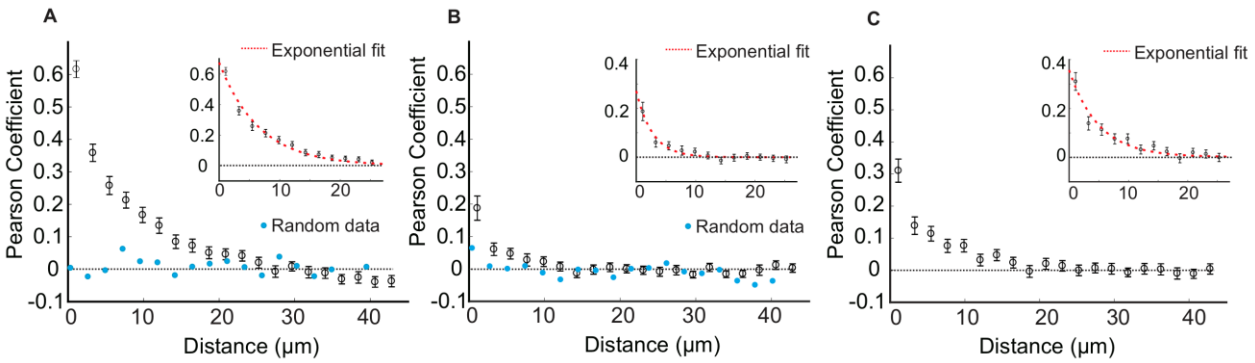

Fig. S6. Spatial correlation of release properties and synaptic size among neighboring boutons, related to Figure 4. The mean ± SEM of spatial correlation coefficients (n = 61 cells) for evoked probability (**A**), spontaneous frequencies (**B**), and Bassoon flat-field corrected intensities (**C**) was plotted for synapses up to 45 μm apart. Blue circles represent the same analysis on random generated synapse locations with random evoked probabilities (**A**) and spontaneous frequencies (**B**), indicating that the observed spatial correlation in empirical data is significant. Insets: single exponential fits to the initial 25 μm results in a length constant of 6.7 μm (95% confidence interval: 5.8, 8.3) in (**A**), 2.7 μm (95% confidence interval: 2.12, 3.8) in (**B**), and 5.0 μm (95% confidence interval: 3.9, 6.8) in (**C**).

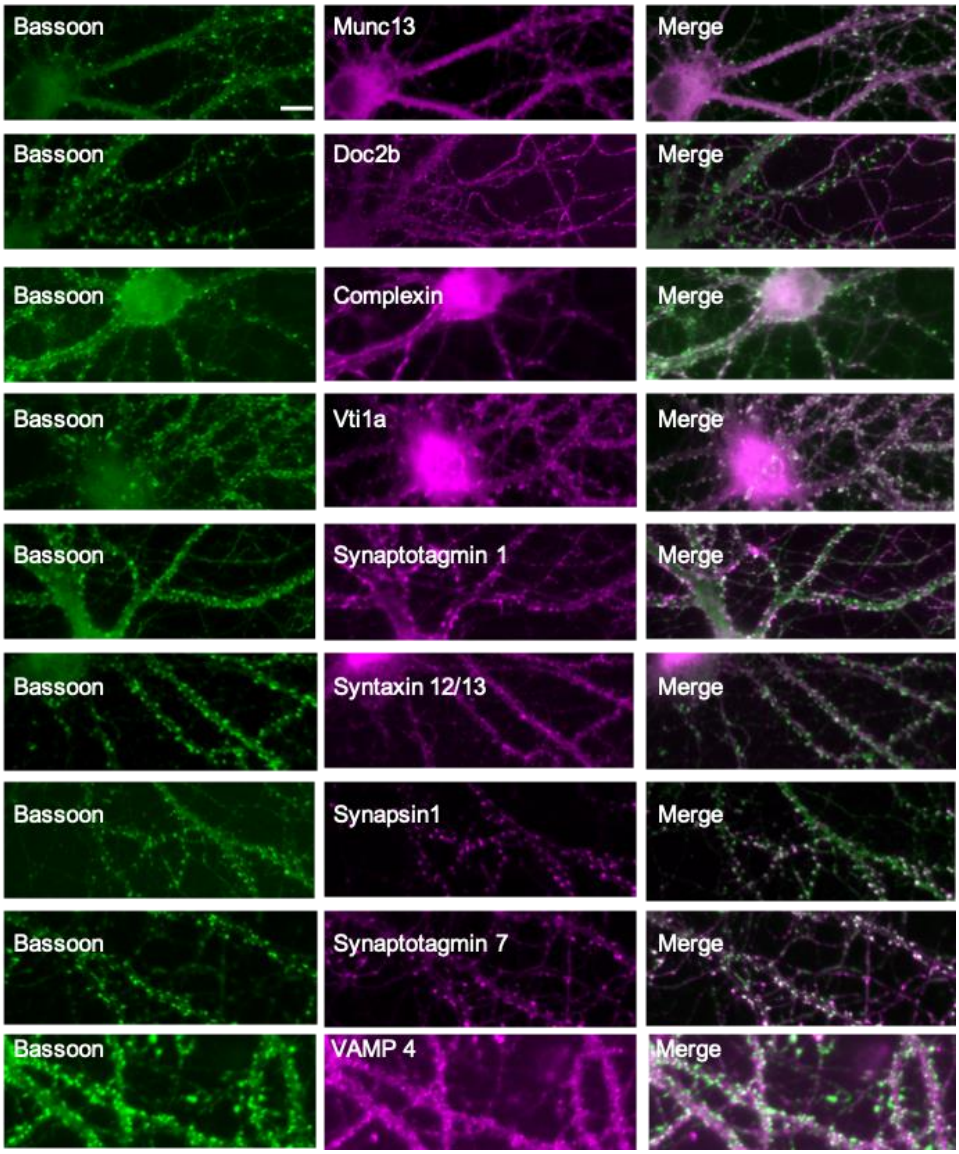

Fig. S7. Expression profile of different proteins at hippocampal synapses, related to Figure 5. Immuno-labeling against Bassoon and different proteins of interest was performed to check for the variability of their relative expression among central synapses. Scale bar 10 µm.

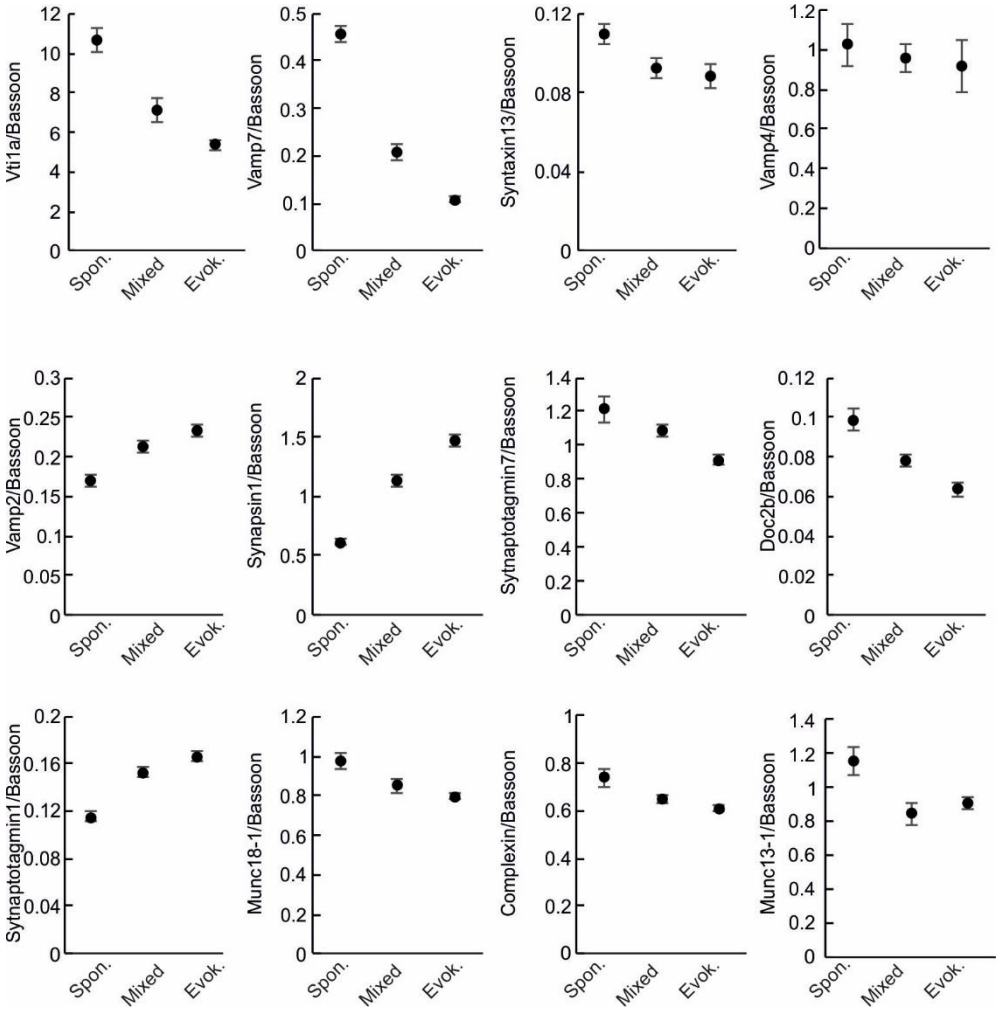

Fig. S8. Relative abundance of proteins in different pools, related to Figure 6. The relative abundance of tested proteins in the 'Mixed' pool was found to be in between the measured abundance in 'spontaneous' and 'evoking' pools. Each graph is from an example cell labeled with the indicated protein. Error bars indicate SEM (n > 50 synapses).

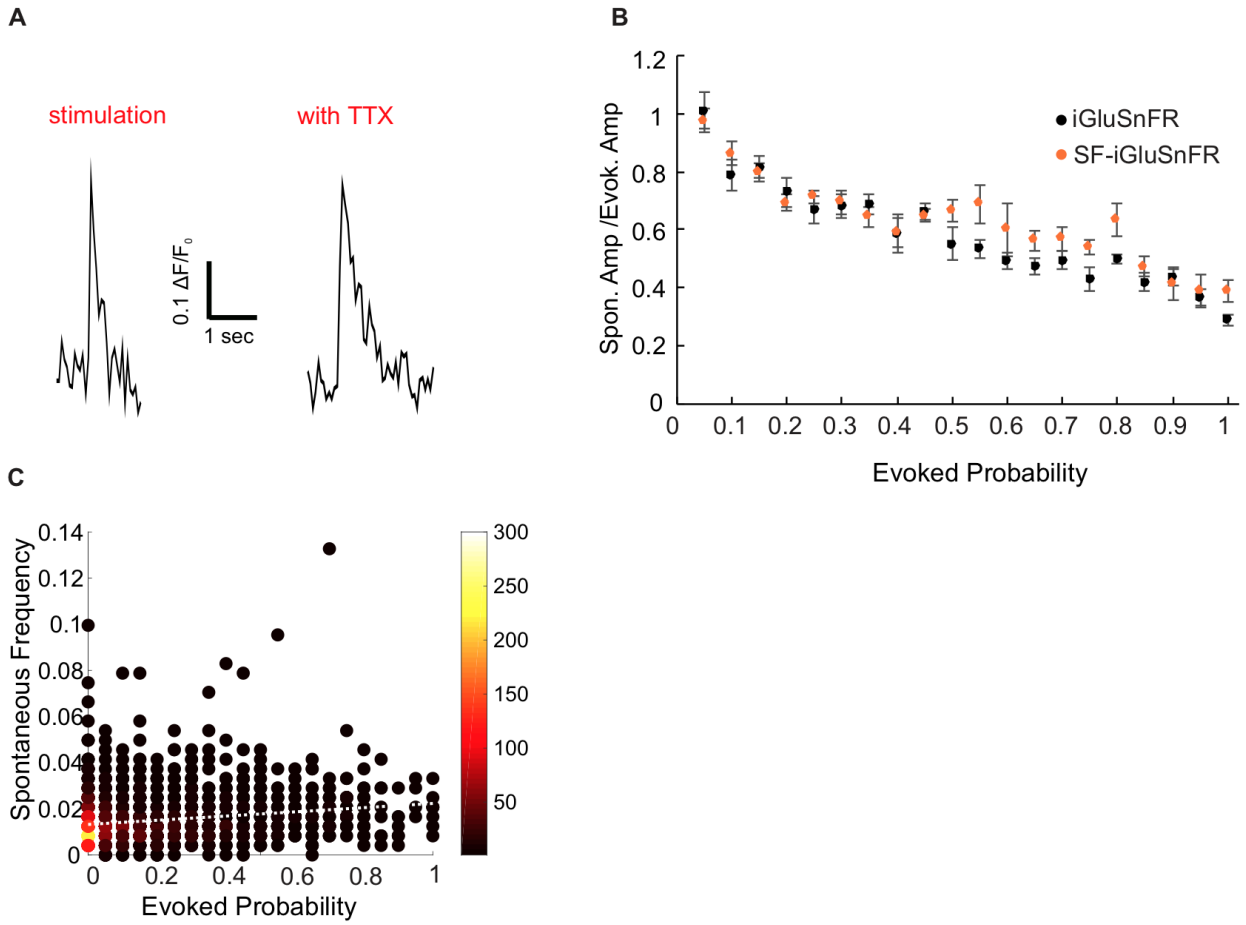

Fig. S9. Characterization of release properties with SF-iGluSnFR, related to figure 2. SF-iGluSnFR is a variant of iGluSnFR in which the circularly permutated eGFP is replaced with circularly permutated superfold (SF) GFP. This variant is reported to improve the *in vivo* imaging of stimulus-evoked glutamate release in comparison with iGluSnFR (Marvin, Scholl et al. 2018). We were able to obtain the same results by repeating the key experiments of our study with SF-iGluSnFR. (**A**) SF-iGluSNFR fluorescence change in response to stimulus-evoked and spontaneous glutamate release (in the presence of TTX) show similar amplitudes. (**B**) Similar to iGluSnFR results, a decrease in ratio of spontaneous amplitude (acquired during the first minute)/evoking amplitude at synapses with higher evoked probabilities was observed, which indicates a higher likelihood of multi-quantal release at these synapses. (**C**) No correlation was observed between the evoked release probabilities and spontaneous frequencies calculated from the measurements with SF-iGluSnFR (R^2 = 0.03), suggesting that these release modes do not share a common mechanism.

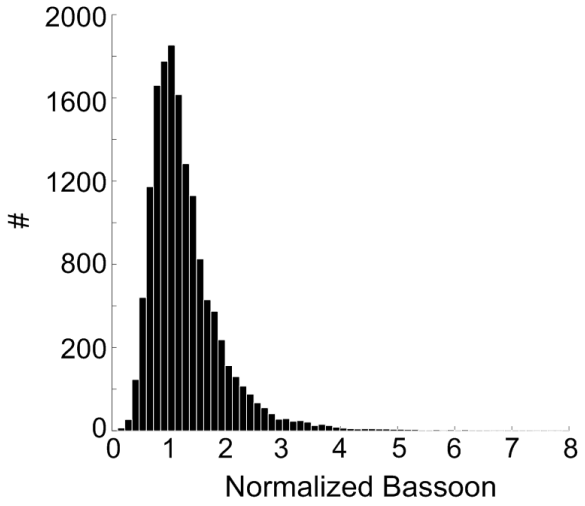

Fig. S10. Distribution of Bassoon intensities, related to Figure 6. In order to compile the Bassoon intensities from all the cells, the flat-filed corrected Bassoon intensities in each cell were normalized to the averaged Bassoon values collected from the synapses of the same cell with low evoked probabilities (< 0.1) and low spontaneous frequencies (< 0.005). The normalized Bassoon values were then pooled and binned into a histogram. An averaged value of 1.3 ± 0.73 (SD) was obtained from the total of ~ 15000 synapses (n > 60 cells).