Supporting Information

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SI Materials and Methods

Primary Cell Culture of Hippocampal Neurons. Hippocampi were dissected from E18 Sprague-Dawley rat embryos, incubated for 15 min with 0.03% trypsin, and dissociated using a fire-polished Pasteur pipette. Cells were diluted in neurobasal medium supplemented with B-27 (Invitrogen) 0.5 mM glutamine, 25 μM glutamate, and 5% FCS and plated on coverslips coated with 0.1% (wt/vol) poly-L-lysine (Peptides International) at a density of 3–6 × 10^3 cm^{-1}. The medium was replaced with serum-free Neurobasal supplemented with B-27 (Invitrogen) 4 h after plating.

Transfections. Cultures of hippocampal neurons were transfected 10–17 days after plating using a calcium-phosphate precipitation protocol. Per 12-mm coverslip, a precipitation mix containing 8 μg of a plasmid containing the cDNA of tdT-Bsn and 16 μg of a plasmid encoding sypH2 as well as 0.25 M $CaCl_2$ was prepared and slowly added to a solution containing (in mM) 274 NaCl, 10 KCl, 1.4 Na_2HPO_4, 15 D-glucose, 42 -(2-hydroxyethyl)-1-piperazineethanesulfonic acid (hepes), pH 7.10. This mix was added to culture dishes in which the medium had been replaced by MEM containing 1/100 volume B-27. Neuronal cultures were incubated with the precipitate for 3–4 h and then washed with a buffer containing (in mM) 144 NaCl, 3 KCl, 2 mM $MgCl_2$, 10 mM hepes, pH 6.70. Cultures were then placed back into their original medium and incubated at 5% CO_2 and 37 °C until used for imaging 2–5 days after transfection.

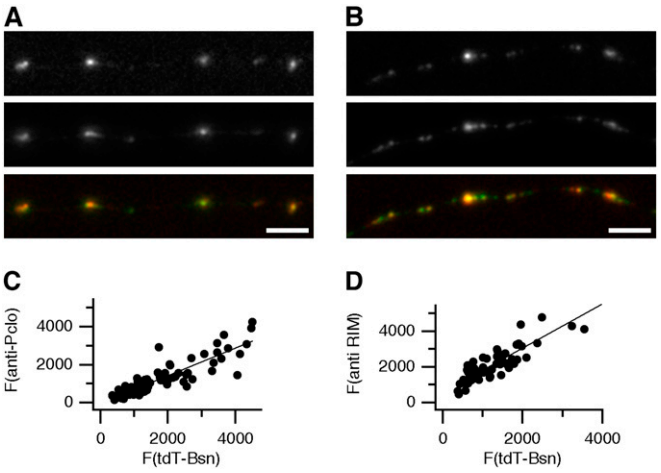


Fig. S1. tdT-Bsn is restricted to active zones and homogeneously distributed within the active zone cytomatrix. (*A*) tdT-Bsn-transfected cultures were subjected to immunocytochemistry using an antibody raised against Piccolo (Pclo). Shown are tdT-Bsn fluorescence (*Top*), Pclo immunofluorescence (*Middle*), and an overlay (tdT-Bsn in red, Pclo immunofluorescence in green). (*B*) tdT-Bsn-transfected cultures were immunostained with an antibody raised against RIM1. Depicted are dT-Bsn fluorescence (*Top*), RIM immunofluorescence (*Middle*), and an overlay (tdT-Bsn in red, RIM immunofluorescence in green). (Scale bars, 5 μm.) (*C*) Correlation between Pclo immunofluorescence and tdT-Bsn fluorescence for 75 synapses in one experiment (r = 0.878). In four other experiments, correlation coefficients ranged between 0.745 and 0.922. (*D*) Correlation between RIM immunofluorescence and tdT-Bsn fluorescence for 62 synapses in one experiment (r = 0.905). Immunocytochemistry on cultures transfected with tdT-Bsn was performed as follows: Cultures were fixed in 4% wt/vol paraformaldehyde, permeabilized with 0.1% vol/vol Triton X-100, and incubated in blocking buffer containing 3% wt/vol BSA before incubation over night at 4 °C with primary antibodies. Antibodies, diluted to their final concentration in blocking buffer, were: anti-Piccolo rabbit polyclonal antiserum (Synaptic Systems) at 1:200 dilution; anti-RIM rabbit polyclonal antiserum (Synaptic Systems) at 1:200 dilution. Primary antibodies were detected using Cy2-labeled secondary antibodies (Jackson ImmunoResearch).

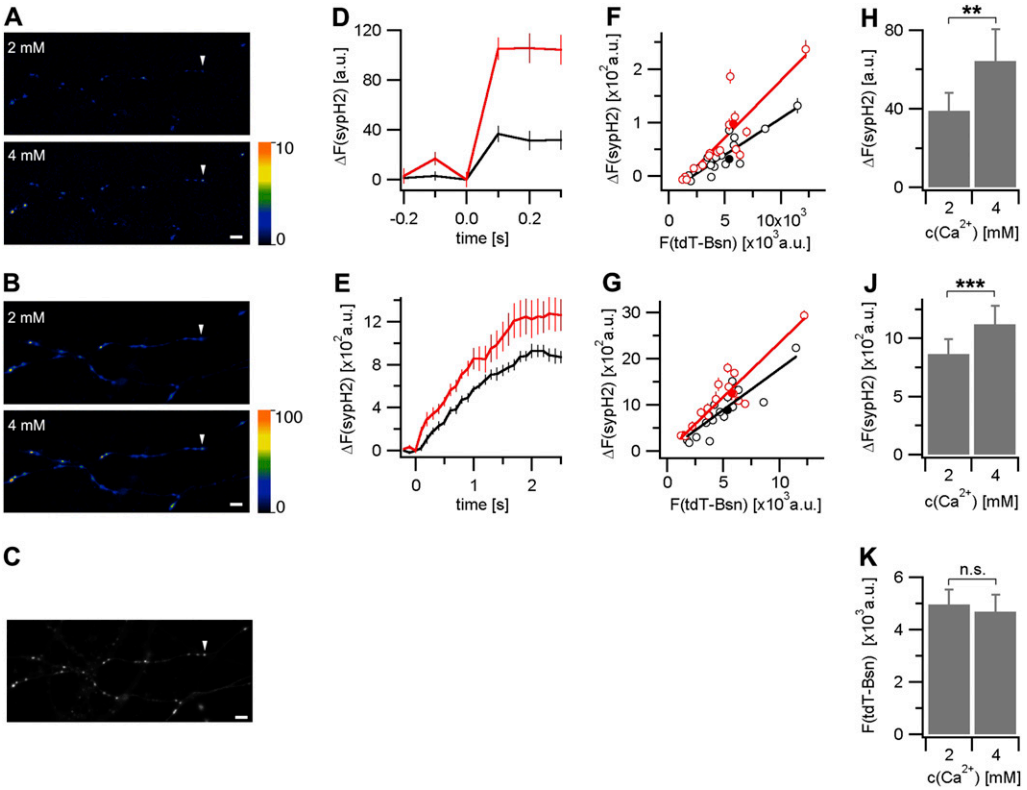


Fig. S2. Correlation of p_r and RRP with active zone size at different extracellular calcium concentrations. (*A*–*C*) Hippocampal neurons in dissociated cultures transfected with sypH2 and tdT-Bsn. SypH2 fluorescence increases to isolated stimulation at 0.2 Hz as well as in response to stimulus trains (40 stimuli at 20 Hz) were measured in the presence of 2 or 4 mM extracellular calcium. Isolated stimuli (90 and 60 at 2 and 4 mM calcium, respectively) and stimulus trains (nine and six trains at 2 and 4 mM calcium), as well as measurements at different $[Ca^{2+}]_o$, were interleaved. Shown are averaged SypH2 fluorescence changes in response to isolated stimuli at 0.2 Hz (*A*) and to stimulus trains of 40 stimuli at 20 Hz (*B*) at 2 mM (*Upper*) and 4 mM (*Lower*) extracellular calcium concentration. tdT-Bsn fluorescence is shown in *C*. (Scale bar, 4 μm.) (*D*) Average sypH2 fluorescence change in response to isolated stimuli at the synapse marked with an arrowhead in *A*–*C*. The fluorescence change at 4 mM calcium (red trace) was significantly higher than that at 2 mM calcium (black trace; $P < 0.001$). (*E*) The sypH2 fluorescence change in response to 40 stimuli at 20 Hz at the same synapse at an extracellular calcium concentration of 4 mM or 2 mM ($P = 0.06$). (*F*) Correlation between averaged sypH2 response to isolated stimulation and tdT-Bsn fluorescence at extracellular calcium concentrations of 2 (black) and 4 mM (red) at all 17 release sites in this experiment. A rise in the extracellular calcium concentration resulted in an increase in the slope of the linear correlation between the measure of p_r and tdT-Bsn fluorescence and a decrease in correlation strength from $r = 0.890$ to $r = 0.857$. (*G*) Correlation between increases of sypH2 fluorescence in response to 40 stimuli at 20 Hz and tdT-Bsn fluorescence. With the rise in extracellular calcium, a slight increase in the slope of the linear correlation as well as its strength ($r = 0.889$ and 0.914) was observed. (*H*) Average sypH2 responses to isolated stimulation increased by 65% with the rise in $[Ca^{2+}]_o$ (** $P < 0.01$, $n = 17$, Wilcoxon Signed Rank test), equivalent to a rise in the average release probability from $p_r = 0.25$ to $p_r = 0.41$. (*I*) The rise in $[Ca^{2+}]_o$ resulted in an increase in the sypH2 response to stimulus trains by 30% (*** $P < 0.001$, Wilcoxon Signed Rank test). In two other experiments in which we altered the extracellular calcium concentration, we observed increases of similar magnitude. This result suggests that the readily releasable pool of synaptic vesicles at synapses between hippocampal neurons is likely not fully mobilized by a train of 40 stimuli at 20 Hz, in line with an earlier report (1). However, the correlation between sypH2 train responses and tdT-Bsn fluorescence slightly increased with increasing calcium concentration in this and similar experiments performed, suggesting that, if anything, we might have underestimated the strength of the correlation between RRP size and active zone cytomatrix size due to this incomplete mobilization. (*J*) The tdT-Bassoon fluorescence at the active zones did not change significantly with the alteration in the extracellular calcium concentration ($P = 0.19$, Wilcoxon Signed Rank test).

1. Moulder KL, Mennerick S (2005) Reluctant vesicles contribute to the total readily releasable pool in glutamatergic hippocampal neurons. *J Neurosci* 25:3842–3850.

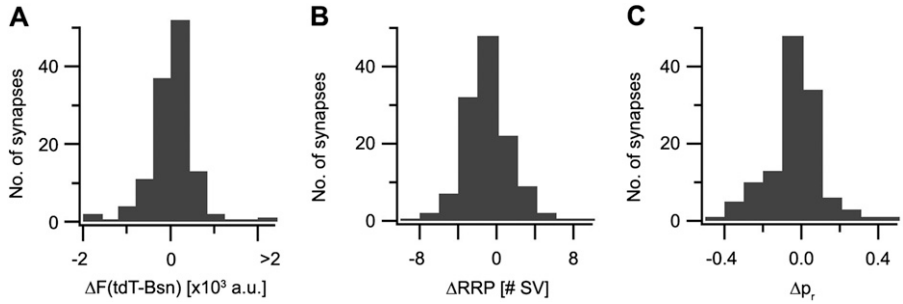


Fig. S3. Distribution of structural and functional presynaptic changes at axospinous synapses between hippocampal neurons in culture. In three experiments, we identified a total of 122 axospinous synapses made by tdT-Bsn and sypH2 transfected neurons after MAP2 and actin staining of fixed cultures. Shown are distributions of changes in tdT-Bassoon fluorescence (*A*), alterations in RRP size (*B*), and release probability (*C*) occurring during the duration of the experiment (5–6 h).

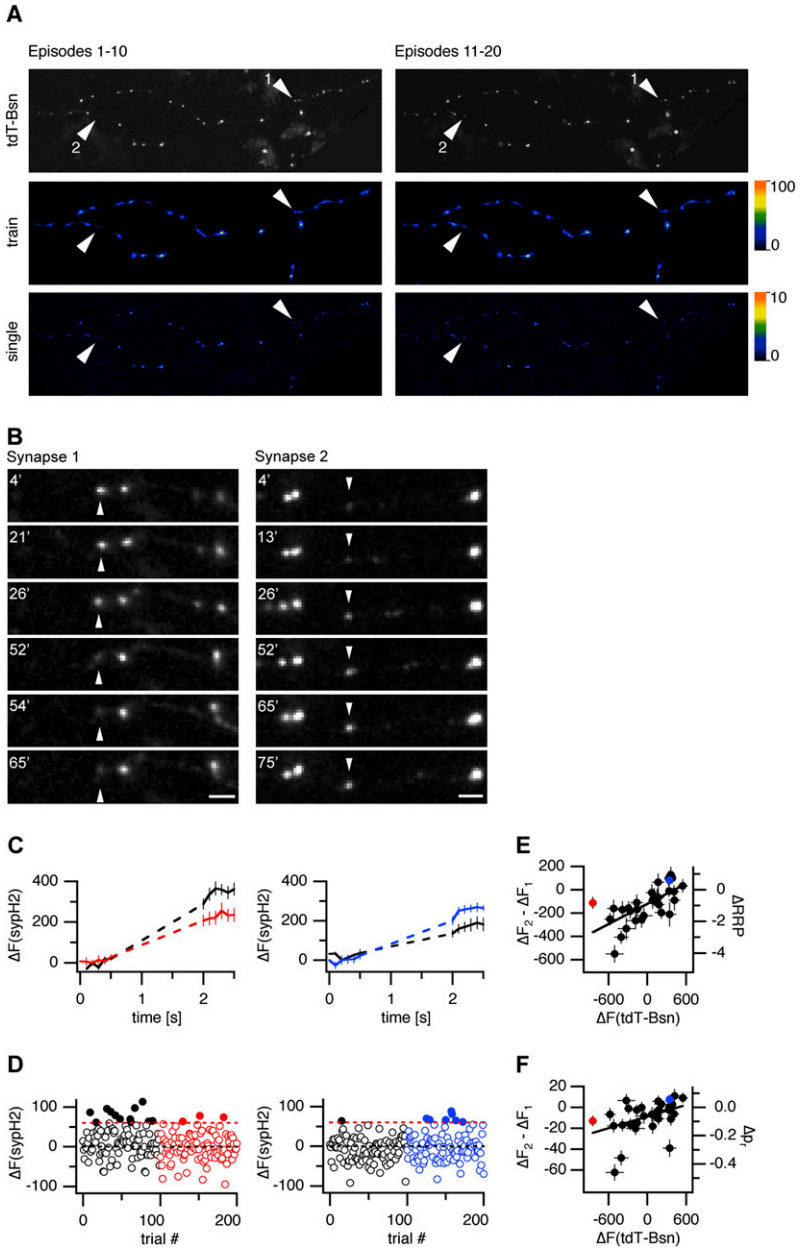
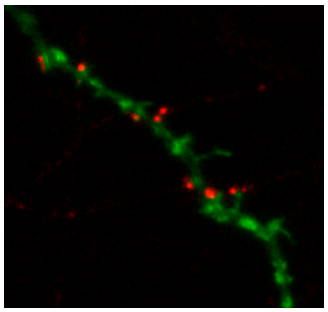


Fig. S4. Alterations of active zone cytomatrix size, RRP size, and release probability occur rapidly. Release sites of a tdT-Bsn and sypH2-expressing neuron were monitored over the course of 75 min to detect any changes in cytomatrix size, RRP size, and p_r. (*A*) Images depict tdT-Bsn fluorescences (*Top*), average increases in sypH2 fluorescence in response to 40 stimuli at 20 Hz (*Middle*), and average increases in sypH2 fluorescence in response to isolated stimuli (*Bottom*) at the onset (0–26 min, *Left*) and end (52-75 min, *Right*) of the experiment. (*B*) Examples of two synapses (arrowheads in *A*) at which the active zone size decreased (*Left*) or increased (*Right*). In the vicinity of these synapses, several mobile tdT-Bassoon clusters are apparent. (Scale bar, 4 μm.) (*C*) SypH2 fluorescence changes at these synapses in response to 40 stimuli at 20 Hz were recorded at the onset (0-26 min, black traces) and end of the experiment (52-75 min, red and blue traces). Decreases and increases in RRP size at synapse 1 and 2, respectively, were statistically significant ($P < 0.01$). (*D*) SypH2 fluorescence changes in response to isolated stimuli at the onset and end of the experiment was significant for synapse 1 ($P = 0.01$), but, because of its very low p_r, failed to reach significance for synapse 2 ($P = 0.12$). (*E*) Correlation between changes in the sypH2 response to stimulus trains delivered at the onset (ΔF_1) and end of the experiment (ΔF_2), respectively, and changes in tdT-Bsn fluorescence for 32 synapses in this experiment ($r = 0.671$, $P < 10^{-4}$). (*F*) Correlation between changes in the average sypH2 response to isolated stimuli delivered at the onset (ΔF_1) and end of the experiment (ΔF_2), respectively, and changes in tdT-Bsn fluorescence for 32 synapses in this experiment ($r = 0.449$, $P = 0.011$).



Movie S1. tdT-Bsn dynamics at axospinous synapses. Movie compiled from the experiment shown in Fig. 3 with 100-fold time compression. tdT-Bsn fluorescence of presynaptic active zones is shown in red and Venus-actin fluorescence of postsynaptic spines and dendritic shaft in green.

Movie S1