Supporting Information

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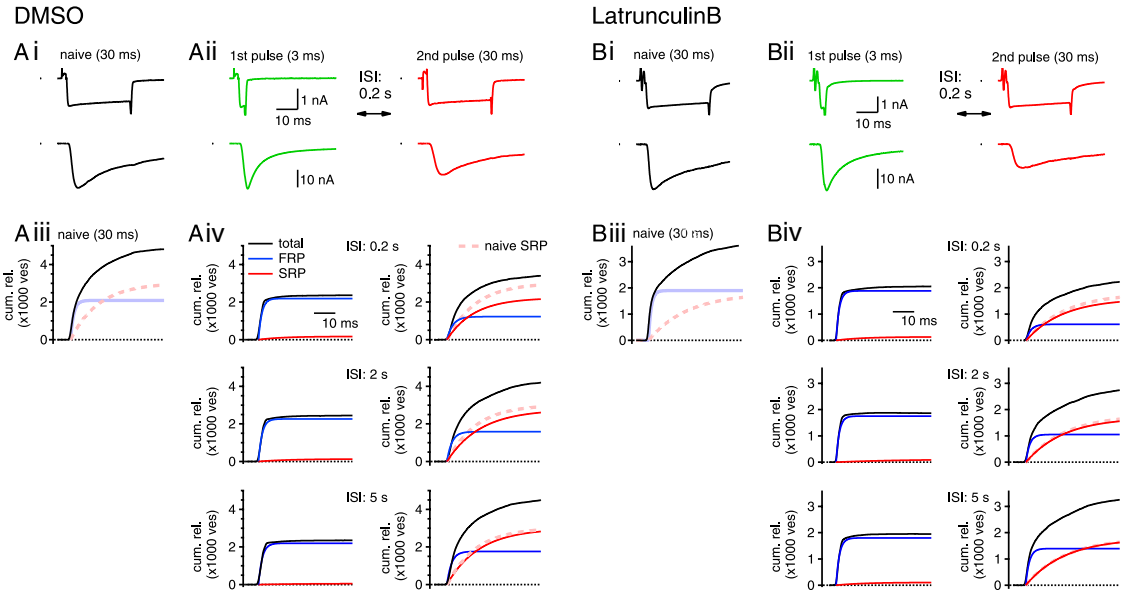

Fig. S1. Recovery of the fast-releasing pool (FRP) after a predepleting pulse of 3 s (preDP3) and concomitant depletion of the slow-releasing pool (SRP). (*A*, *i* and *ii*) Presynaptic Ca^{2+} current (*Upper*) and EPSC (*Lower*) evoked by a depleting pulse of 30 ms (DP30) [naive30 ms (DP30)] (*A*, *1*) and dual-pulse protocol [preDP3 followed by a DP30, interstimulus interval (ISI) = 0.2 s] (*A*, *ii*) at the synapse shown in Fig. 3*A*, *i*. (*iii* and *iv*) Cumulative quantal release (black trace) evoked by a naive DP30 (not preceded by a preDP; *A*, *iii*) and by a DP30 after a preDP3 with different ISIs (*iv*) (*Top*, 200 ms; *Middle*, 2 s; *Bottom*, 5 s) at the same synapse. Fast (blue traces) and slow (red traces) components of a biexponential fit to the cumulative release are shown. Although the SRP (red trace) was hardly released by the preDP3 at the ISI of 0.2 s (*iv*, *Left*), the size of SRP at the second pulse (red trace, *iv*, *Right*) was smaller than that measured in the naive state (broken pink line). (*B, i–iv*) Similar experiments were done with 15 μM latrunculin B (latB) included in the presynaptic pipette (the same synapse shown in Fig. 3*A*, *ii*). Note that with an ISI of 0.2 s (*B*, *iv*) there is little difference in SRP size between the naive state (pink broken line) and after a preDP3 (red trace).

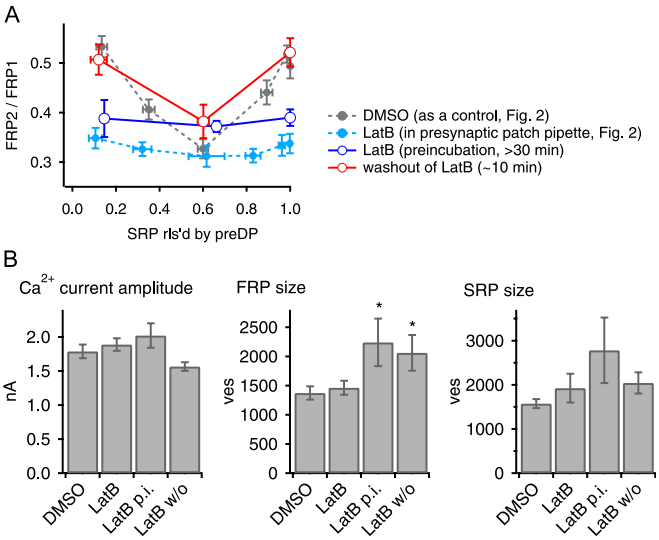

Fig. S2. Reversibility of the effects of latB on SRP-dependent recovery (SDR) and Ca^{2+}-dependent recovery (CDR). (*A*) Experiments similar to that shown in Fig. 2 were done using a slice preincubated for 30 min in artificial cerebrospinal fluid (aCSF) containing 15 μM latB before (blue trace, $n = 3$) and after 10 min washout (red trace) of latB ($n = 4$). Both CDR and SDR were suppressed by pretreatment with latB and were restored after the wash-out of latB. For comparison, dotted curves are results in Fig. 2*B*. (*B*) For unknown reasons, FRP and SRP sizes were significantly higher in the calyx synapses pretreated with latB. The increased size of the FRP pool was not reversed by the washout of latB. $*P < 0.05$.

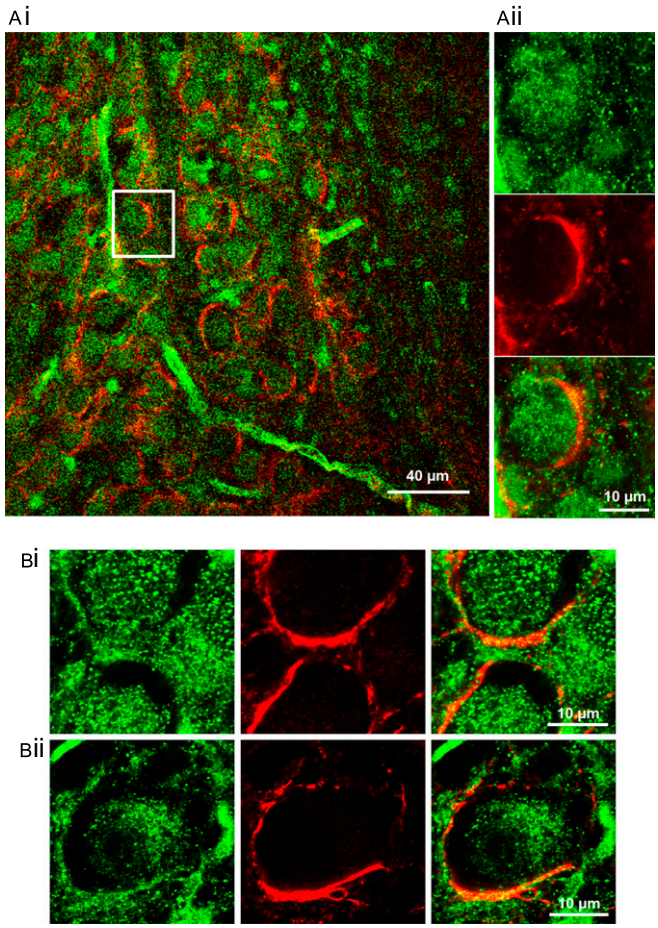

Fig. S3. Colocalization of nonmuscle myosin II and synaptophysin in the calyx terminal. (*A*) Immunofluorescence images of the rat medial nucleus of the trapezoidal body stained with anti-nonmuscle myosin II (green) and anti-synaptophysin (red) antibodies. The box in the low-power image (*A, i*) is shown in the high-resolution immunofluorescence images (*A*, *ii*) of nonmuscle myosin II (*Top*) and synaptophysin (*Middle*) staining and in the overlaid image (*Bottom*). Note that green spots are present not only in the postsynaptic cells but also in the synaptophysin-immunoreactive calyx-shaped presynaptic terminals. (Scale bars: 40 μm in *A*, *i*; 10 μm in *A*, *ii*.) (*B*, *i* and *ii*) Other calyx synapses where the postsynaptic cells shrank or are partly absent but the presynaptic terminals are relatively preserved. Despite the damage to the postsynaptic cell, the myosin-immunoreactive spots are evident in the presynaptic terminals. Methods: Sprague–Dawley rats (postnatal day 9; $n = 3$) were killed by decapitation, and the brain was placed in low-calcium aCSF. After the dura and pia were removed locally, the hindbrain was cut off and immersion-fixed in PBS containing 4% (wt/vol) paraformaldehyde for 24 h at 4 °C. After fixation, the brainstem was washed three times with PBS for 30 min at 4 °C. Transverse brainstem slices (50 μm thick) were made with a vibratome and were immersed in PBS. Next, slices were incubated in PBS containing 0.5% (vol/vol) Triton X-100 for 20 min at room temperature and then were transferred to blocking solution [5% (vol/vol) donkey serum, 0.1% Triton X-100 in PBS] for 2 h. Subsequently slices were incubated with primary antibodies in blocking solution for 24 h at 4 °C. The primary antibodies were rabbit polyclonal anti-nonmuscle myosin IIA diluted 1:500 (ab24762; Abcam) and mouse monoclonal anti-synaptophysin diluted 1:400 (S5768, Sigma). After incubation, slices were washed three times for 10 min with the PBS containing 0.1% Triton X-100 and were incubated with secondary antibodies in the blocking solution for 1 h. The secondary antibodies were Alexa Fluor 488-conjugated anti-rabbit antibody diluted 1:100 (A21206; Invitrogen) and Cy5-conjugated anti-mouse antibody diluted 1:200 (715-175-150; Jackson ImmunoResearch). After slices were rinsed three times for 10 min with the PBS containing 0.1% Triton X-100, they were mounted on microscope slides. Fluorescence signal was visualized by the confocal microscopy (DMIRE2; Leica,) using a 63× oil-immersion objective. (Scale bars, 10 μm.)

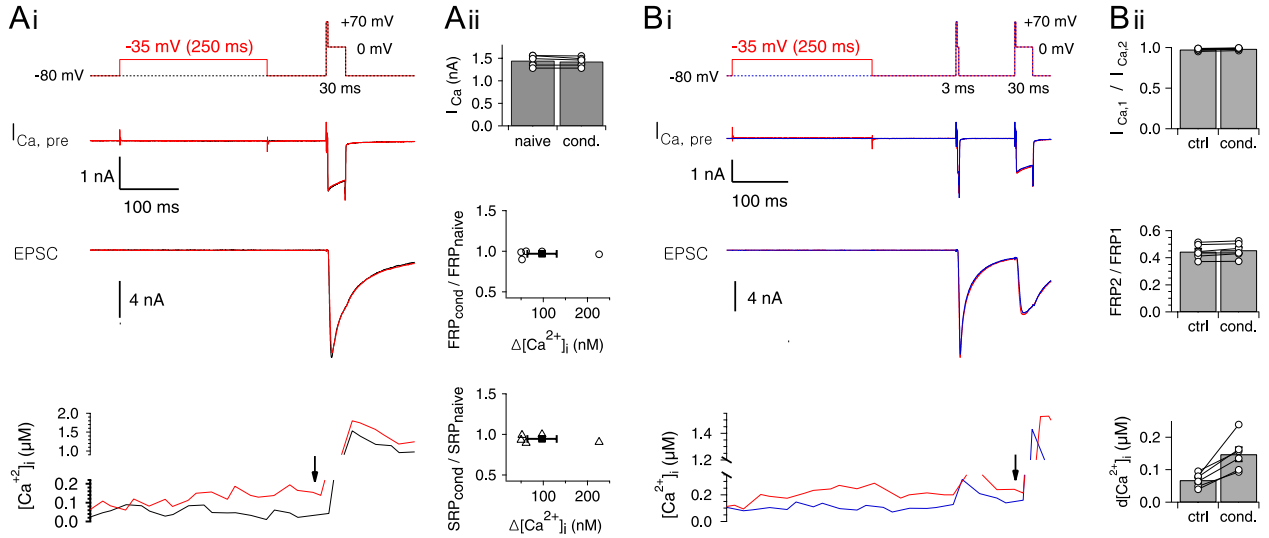

Fig. S4. A small elevation of resting $[Ca^{2+}]$ had little effect on the estimates of FRP and SRP. (*A*, *i*) Pulse protocols for a DP30 alone (naive DP30, black) and the DP30 preceded by predepolarization to −35 mV (conditioned DP30, red) are superimposed (*Top*). Corresponding traces for presynaptic Ca^{2+} currents (*Second Row*), excitatory postsynaptic currents (EPSCs) (*Third Row*), and presynaptic Ca^{2+} transients (*Bottom*) are shown using the same color code. For presynaptic $[Ca^{2+}]_i$ measurement, 0.45 mM EGTA and 50 μM Fura-4F were included in the presynaptic pipette solution. (*A*, *ii*) Mean values for the amplitude of presynaptic Ca^{2+} current evoked by a naive DP30 and a conditioned DP30 (*Top*). The size of an FRP (*Middle*) or an SRP (*Bottom*) released by a conditioned DP30 relative to that released by a naive DP30 is plotted as a function of presynaptic Ca^{2+} increments induced by predepolarization. Filled squares indicate mean values. Note: The FRP and SRP sizes are not significantly dependent on the resting $[Ca^{2+}]_i$ level up to 200 nM. The arrow in *A*, *i* indicates the time point of $[Ca^{2+}]_i$ estimation. (*B*, *i* and *ii*) As in *A*, except that the DP30 was preceded by a preDP3 to induce a partial depletion of FRP (ISI = 100 ms). (*i*). Pulse protocols for the estimates of FRP size after a preDP3 with (red) or without (blue) a subthreshold predepolarizing pulse (to −35 mV for 250 ms) (*Top*). The same color code is used in lower rows. (*ii*) Mean values for the ratio of presynaptic Ca^{2+} current amplitude (*Top*) and recovered fraction of the FRP (*Middle*) with (cond) or without (ctrl) a subthreshold depolarization. The subthreshold predepolarization elevated $[Ca^{2+}]_{rest}$ from 68 ± 9 nM to 148 ± 22 nM (*Bottom*; $n = 6$). Note that the estimate of the recovered FRP size was not affected by elevation of $[Ca^{2+}]_i$. Error bars indicate SEM.

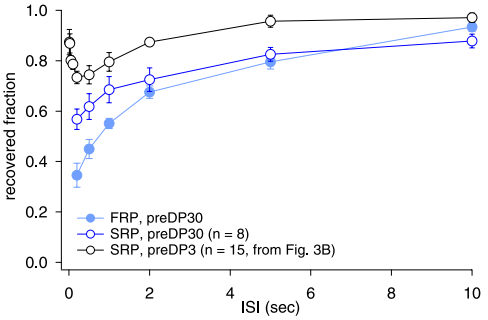

Fig. S5. The recovery time courses of synaptic vesicle pools after a preDP3 (black circles) or after a preDP30 (blue symbols). The data for SRP recovery after a preDP3 are from Fig. 3*B*, *ii*.

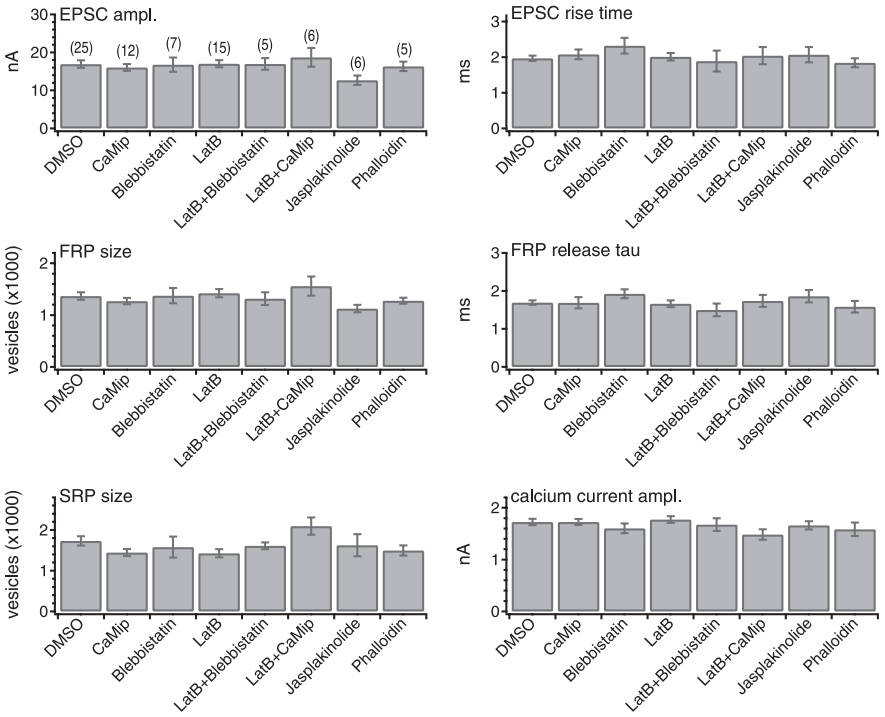

Fig. S6. Effects of drugs used in the present study on the baseline parameters of EPSC. Summary bar graphs for amplitude and rise time of the baseline EPSC amplitude (*Top*), the FRP size and fast-release time constant (*Middle*), and the SRP size and presynaptic Ca^{2+} current amplitude elicited by the preDP30 (*Bottom*). Data are from the synapses in Figs. 2 and 3. No parameters were significantly different from control values (DMSO). Values are shown as mean ± SEM.

Table S1. Mean values for the recovered fraction of the FRP size 750 ms after preDPs of different duration

		Recovered fraction of the FRP after a preDP (%)		
Conditions	*n*	preDP3	preDP10	preDP30
DMSO (1/1000)	10	53.3 ± 2.1	32.7 ± 1.9	51.8 ± 1.7
Calmodulin inhibitor peptide (20 μM)	7	53.2 ± 2.1 ($P = 0.97$)	31.6 ± 1.9 ($P = 0.71$)	35.3 ± 2.2 ($P < 0.01$)
Latrunculin B (15 μM)	6	34.8 ± 2.1 ($P < 0.01$)	31.2 ± 2.2 ($P = 0.63$)	33.4 ± 2.1 ($P < 0.01$)
Blebbistatin (100 μM)	7	36.7 ± 1.2 ($P < 0.01$)	29.8 ± 1.9 ($P = 0.33$)	35.8 ± 1.4 ($P < 0.01$)
Jasplakinolide (1 μM)	6	51.4 ± 1.6 ($P = 0.53$)	35.8 ± 1.1 ($P = 0.25$)	47.9 ± 1.6 ($P = 0.16$)
Phalloidin (20 μM)	5	53.0 ± 2.5 ($P = 0.93$)	36.4 ± 1.3 ($P = 0.22$)	51.9 ± 1.6 ($P = 0.96$)
Latrunculin B + calmodulin inhibitor peptide	6	37.8 ± 2.1 ($P < 0.01$)	29.5 ± 0.9 ($P = 0.25$)	36.1 ± 2.6 ($P < 0.01$)
Latrunculin B + blebbistatin	5	33.4 ± 3.0 ($P < 0.01$)	31.1 ± 2.6 ($P = 0.64$)	32.5 ± 2.7 ($P < 0.01$)

Statistical data are shown as mean ± SE. preDP3, preDP of 3 ms duration; preDP10, preDP of 10 ms duration; preDP30, preDP of 30 ms duration. *n*, number of synapses studied. *P* values were calculated from Student's *t* test. Also see Fig. 2.