Supplementary Methods

Slice preparation and electrophysiology

Transverse brainstem slices were cut from young Wistar rats (P8-10), as described previously. During recordings, slices were superfused with an extracellular solution containing (in mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 25 dextrose, 1.25 NaH₂PO₄, 0.4 ascorbic acid, 3 myo-inositol, 2 sodium pyruvate, and 25 NaHCO₃, pH 7.4 when bubbled with 95% O₂ and 5% CO₂, at room temperature (22-24 °C). Simultaneous pre- and postsynaptic voltage-clamp recordings were performed using two patch-clamp amplifiers (Axopatch 200 A and B, respectively, Axon Instruments, CA). The pre- and postsynaptic holding potential was −80 mV. The postsynaptic pipette solution contained (in mM): 115 cesium gluconate, 20 CsCl, 10 phosphocreatine disodium salt, 4 MgATP, 0.3 GTP, 0.5 EGTA, and 10 HEPES, pH 7.2 adjusted with CsOH. Postsynaptic access resistance was 13.2 ± 0.7 MΩ (n = 54), and was electronically compensated to 90-95% (lag ≤ 10 µs). NMDAR-mediated currents were blocked using D-APV (50 µM, Tocris, UK) and glycine-mediated currents were inhibited by strychnine (5 µM)². AMPA-current desensitization was minimized by application of cyclothiazide (CTZ, 100 µM, RBI, MA) if not stated otherwise. When laser-evoked EPSCs were compared to AP-evoked release, the intact presynaptic terminal was stimulated using a bipolar electrode placed at the mid-line of the brain slice.

Ca²⁺ uncaging and rapid fluorescence detection

Presynaptic terminals were dialyzed with solutions containing (in mM): K-gluconate (30-80), KCl (20), Heps (20), Na₂-ATP (5.0), MgCl₂ (2.0), GTP (0.5). Each experimental day, CaCl₂, DM-nitrophen (Calbiochem, CA), EGTA and BAPTA were added at various concentrations (see legend of Fig. 1), and pH and osmolality were adjusted to 7.2 and 300 mosm/kg. The low-affinity Ca²⁺ indicator Oregon Green-BAPTA-5N (1 mM; Molecular Probes, OR) was used to measure rapid [Ca²⁺] transients, which was calibrated in synaptic terminals using the same composition as in physiological recordings, but with highly buffered Ca²⁺ at three concentration values. Because fluorescence levels measured from different terminals vary because
of volume differences, fluorescence values were normalized to the accessible volume of the terminal, estimated using the loading time constant and the access resistance of each recording. The $F_{\text{max}}/F_{\text{min}}$ ratio and the $K_d$ of OGB-5N were determined \textit{in situ} to be 11.9 and 41 µM, respectively.

Rapidly decaying $[\text{Ca}^{2+}]$ transients were evoked by partial photolysis of DM-n by UV pulses from one or two Nd:YAG lasers (Continuum Minilite 2, CA), which could be triggered independently to permit inter-stimulus intervals down to 5 ms. The pulses were coupled into the epifluorescence port (TILL photonics, Germany) of an upright microscope (Axioskop FS2, Zeiss, Germany) using quartz light guides (TILL photonics) and combined using a beam-splitter (T50/R50, AHF, Germany). Excitation light (485 nm) from a monochromator (TILL photonics) was combined with the UV pulses by a second beam splitter (T70/R30; AHF). The pulse energy was attenuated using an analyzer plate and neutral density filters. UV pulses were delivered to the specimen and fluorescence was collected using a water immersion objective lens (LUMPlanFl 60x, Olympus, Japan). Two adjustable field stops in the epifluorescence port restricted the area in the focal plane illuminated by the laser pulses and the fluorescence excitation to a square of 30 µm side length. The spatial homogeneity of the laser pulse energy and monochromator intensity was confirmed using a CCD camera to image thin layers of fura-2 in the focal plane, which was excited by a laser pulse or the monochromator, respectively.

$[\text{Ca}^{2+}]$ transients in presynaptic terminals were measured using OGB-5N (1 mM). Fluorescence transients from a circular region encompassing the entire presynaptic terminal were recorded using a PIN photodiode (S5973, Hamamatsu, Japan) mounted in a custom-made low noise housing and amplified with a patch-clamp amplifier (Axopatch 200B). Background fluorescence from spilled OGB-5N was measured from the region of interest when the presynaptic pipette was in the cell-attached configuration and a stable background level had been achieved (>10 min.). After background subtraction, fluorescence transients were expressed as $\Delta F/F$ transients. To prevent saturation of the photodetection circuit the ‘blank activate’ circuit of the amplifier was activated for 260 µs starting 180 µs before the UV pulse. This procedure prevented flash artifacts in the recording, which was confirmed by the
absence of changes in the photocurrent when UV pulses were delivered onto a solution containing DM-n and OGB-5N, but no CaCl$_2$. The photo-current and electrophysiological recordings were filtered at 5 kHz (4-pole Bessel) and sampled at 50 kHz (ITC16, Instrutech, NJ).

Homogeneous [Ca$^{2+}$] transients were measured using the low affinity Ca$^{2+}$ indicator OGB-5N. Even a low affinity Ca$^{2+}$ indicator, however, does not track the [Ca$^{2+}$] time course within ~0.2 ms after photolysis because of its finite Ca$^{2+}$ binding kinetics, and the recorded fluorescence signal represents a low-pass filtered version of the [Ca$^{2+}$] transient time course. Therefore, detailed modeling of the [Ca$^{2+}$] relaxation was used to reconstruct the time course of the [Ca$^{2+}$] (see below). We also tested the possibility that a very brief (<0.2 ms), but large overshoot in [Ca$^{2+}$] occurred, which was not reported by the [Ca$^{2+}$] relaxation model, but might have influenced the amount and time course of release. Thus, we triggered release with paired [Ca$^{2+}$] transients evoked by a laser pulse (duration ~5 ns) and a flash lamp (flash duration >1 ms) at ISIs of 50 or 100 ms in the same synapse, evoking $\Delta F/F$ transients of similar amplitude (not shown). EPSCs evoked this way were very similar, arguing against a significant influence of an initial [Ca$^{2+}$] overshoot on the magnitude or time course of the EPSC.

**Analysis**

Temporal parameters of glutamate release were quantified by taking the EPSC 20-80% rise time and time-to-peak as reporters of the time window of increased release probability. Rise times and time to peak were measured from least-squares fits to the EPSC using a function of the form:

$$f(t) = A (1+\text{Erf}[k_1(t-t_0)]) \times [e^{-k_2(t-t_0)} + k_4 e^{-k_3(t-t_0)}],$$

in order to minimize variability in the individual measurement due to quantal noise, especially in small EPSCs. EPSC delays were taken as the interval between the time of the UV pulse and the time when the EPSC crossed a threshold of ~30 pA. Experimental data were analyzed and fit with the least-squares algorithm using Igor 4.0 (Wavemetrics, OR). When calculating the least-squares fit curve and the 1 S.D. prediction bands of the dependence of EPSC rise time and time-to-peak on $\Delta F/F$ half-
width, a straight line was fit to the sqrt/sqrt-transform of the data in order to reduce heteroscedasticity (Fig. 2d,e).

When analyzing paired [Ca\(^{2+}\)] transients, the two \(\Delta F/F\) peak values of a pair of \(\Delta F/F\) transients were measured from baseline level before the first flash, whereas EPSC amplitudes were measured from the negative peak of the EPSC to the postsynaptic current level immediately preceding the first and the second flash, respectively. When paired \(\Delta F/F\) transients were evoked at ISIs of 20, 50 and 100 ms, the second \(\Delta F/F\) transient had a 11 ± 1.6% (\(n = 63\) sweeps) larger half-width, because less free Ca\(^{2+}\) buffer was available during the second pulse. In order to correct for the bias arising from the approximately linear dependence of EPSC amplitudes on the \(\Delta F/F\) half-width, the EPSC amplitude ratio was divided by the half-width ratio of a given pair of \(\Delta F/F\) transients before it was plotted against the peak ratio of the same pair of transients (Fig. 5e-g). No such correction was done for ISI = 5 ms. For ISIs of 20, 50 and 100 ms, only sweeps were included in which the first \(\Delta F/F\) transient had decayed to <5% of its peak level by the time of the second pulse (Fig. 5e-g). For ISIs of 5 ms, only sweeps were included in which the first \(\Delta F/F\) transient had decayed to not less than 25% of its peak level by the time of the second pulse (Fig. 5h). Data are presented as mean ± SEM, if not stated otherwise.

Simulations
A kinetic model of the relaxation of the [Ca\(^{2+}\)] following partial photolysis of DM-n was devised in order to back-calculate the [Ca\(^{2+}\)] time course from measured \(\Delta F/F\) traces. The model took into account the presence of multiple Ca\(^{2+}\) buffers in the solution (DM-n, OGB-5N, EGTA, BAPTA, ATP) and the interaction of DM-n and ATP with Mg\(^{2+}\) (Supplementary Fig. 1 online). To account for the observation that Ca\(^{2+}\)-dependent fluorescence changes of OGB-5N exhibited at least two temporal components, DM-n was modeled to decay along two parallel pathways at different rate constants (\(k_{\text{phot1}}, k_{\text{phot2}}\); Supplementary Table 1 online). DM-n intermediates in the first pathway decayed to two photoproducts of identical, low affinity for both Ca\(^{2+}\) and Mg\(^{2+}\) (photoproduct DM\(_1\)), whereas those in the second pathway decayed to equimolar fractions of two photoproducts (photoproduct DM\(_1\) and DM\(_2\), respectively),
which accounted for the relatively high buffer capacity of photoproducts observed in vitro. The slower decay of \( \Delta F/F \) transients in the calyx was attributed to the action of an endogenous \( \text{Ca}^{2+} \) buffer in the terminal and included in the model. From the concentration time course of the indicator-\( \text{Ca}^{2+} \) complex, we calculated the predicted \( \Delta F/F \) time course, which was convolved with the measured impulse response of the photodetection system before it was compared to measured \( \Delta F/F \) traces. Modeled \( \Delta F/F \) transients were overlaid with the measured transients and concentration and rate constants of the endogenous buffer and the fraction of DM-n photolyzed were adjusted manually until the model matched the experimental record (5 cuvette and 5 calyx transients for each of 7 different compositions of the presynaptic solution, see legend to Fig. 1). Because several \( \text{Ca}^{2+} \) buffers contributed to the decay of the [\( \text{Ca}^{2+} \)] transients in parallel, rate constants could not be determined with high accuracy. However, while the predicted \( \Delta F/F \) was invariant to some simultaneous changes in pairs of model parameters, so was the predicted [\( \text{Ca}^{2+} \)] time course, indicating that the model is a good reporter of the [\( \text{Ca}^{2+} \)] time course, which is used to calculate predictions from a second model describing the \( \text{Ca}^{2+} \) sensor of release.

The \( \text{Ca}^{2+} \) sensor model was the same as described previously in Ref. 3, with 5 \( \text{Ca}^{2+} \) binding sites of equal affinity and a \( \text{Ca}^{2+} \)-independent activation and deactivation rate constant of the fully occupied sensor (see Supplementary Fig. 3 online). The same rate constants and pool size were used except for a \( \text{Ca}^{2+} \) association rate constant (\( \alpha \)) reduced by 20% (\( \alpha = 0.24 \, \mu\text{M}^{-1}\text{ms}^{-1} \); dissociation rate constant \( \beta = 3 \, \text{ms}^{-1} \); activation rate constant \( \gamma = 30 \, \text{ms}^{-1} \); deactivation rate constant \( \delta = 8 \, \text{ms}^{-1} \); maximal release rate constant \( \rho = 40 \, \text{ms}^{-1} \)). This modification was required to fit the data in Fig. 2f and Fig. 3e. This adjustment is not unexpected given that a different \( \text{Ca}^{2+} \) indicator was calibrated de novo in the calyx of Held. Release rates predicted by the model were convolved with the waveform of the average miniature EPSC measured previously\(^3\) to calculate the time course of the compound EPSC. Monte Carlo simulations of the time course of individual EPSCs were performed by calculating the instantaneous release probability in time bins of 2-20 \( \mu\text{s} \) and comparing its value to values obtained from a random number generator in Igor 4.0. All other simulations were implemented in Mathematica 4.1 (Wolfram Research, IL).
References in Supplementary Methods


