SUPPLEMENTAL METHODS

Comparing FRAP of different fluorophores

To compare YFP and Alexa-Fluor 594 FRAP measurements, YFP-expressing pyramidal cells were filled with Alexa-Fluor 594 through a patch pipette. Two-photon FRAP experiments were performed at 920 nm, and the recovery of both fluorophores in individual spines was recorded in separate color channels. In a sample of 30 spines, the FRAP time constants τ_{YFP} and $\tau_{Alexa\text{-}Fluor}$ were correlated and well fit by a linear function:

$$\tau_{YFP} = 4.85 \cdot \tau_{Alexa\text{-}Fluor}$$

Thus, we multiplied the time constants measured in rat hippocampus by 4.85 to compare them with our measurements in YFP-expressing mice *in vivo* and in acute slices (Fig. 6C).

Estimation of spine volume

Assuming homogenous distribution of Alexa-Fluor 594 in the cytoplasm, the integrated fluorescence intensity of a spine is proportional to its cytoplasmic volume (Svoboda, 2004; Holtmaat et al., 2005). For each cell, a calibration measurement was taken by focusing the laser into the proximal apical dendrite, a cellular compartment large enough to contain the entire point-spread function (PSF) of our microscope, to obtain the maximum fluorescence intensity (f_{max}) from this particular cell. This calibration measurement was typically taken at a different depth (z_{cal}) than the spine image (z_{spine}), and we had to correct for attenuation of the laser by an experimentally determined attenuation function: $f(z) = f_{max}*\exp(a*(z_{cal}-z_{spine}))$, with $a = -0.015\ \mu m^{-1}$ (attenuation coefficient). To measure the absolute volume of a spine (V_{spine}), we first calculated the Gaussian intensity distribution a hypothetical PSF-sized object would produce if imaged at the same zoom factor and the same depth than the spine (f_{sim}). The integrated intensity of f_{sim} (sum of all pixel values within a region of interest, $\iint f_{sim}$) was then compared to the integrated intensity of the spine

image in a maximum intensity z-projection ($\iint f_{spine}$). The volume of the spine is related to the spine intensity as follows:

$$V_{spine} = \iint f_{spine} * V_{PSF} / \iint f_{sim}$$

The volume of the PSF (V_{PSF} = 0.38 µm^3) was determined using fluorescent beads (0.1 µm, Molecular Probes).

NEURON spine model

We used the NEURON simulation environment (Hines and Carnevale, 1997) to model a single dendritic spine of 0.11 µm^3 volume (average volume of all functional spines in our sample), equipped with NMDARs, AMPARs, and voltage-gated calcium channels. To ensure a realistic EPSP time course in the spine head, we connected the spine through a thin neck (R_{neck} = 1.2 GΩ) to the dendrite of a multi-compartment model of a CA1 pyramidal cell taken from the NEURON database (Golding et al., 2001). Active conductances were removed from soma and dendrites. The membrane time constant (τ_m) was 30 ms (R_m = 40 kΩ cm^2, C_m = 0.75 µF/cm^2). The original model had no spines, but was compensated for the presence of spines by increasing C_m by a factor of 2 and decreasing R_m by a factor of 2 beyond 100 µm from the soma. An intracellular resistivity of R_i = 150 Ω cm and a membrane resting potential of V_{rest} = -65 mV were used.

The kinetic equations for the AMPA and NMDA mechanism were taken from (Franks et al., 2002). The synaptic AMPA current I_{AMPA} was calculated as $I_{AMPA} = g_{AMPA}(V - E_{AMPA})$, where g_{AMPA} is the total conductance, V is the membrane potential, and E_{AMPA} is the reversal potential of AMPARs. The time course of the AMPA conductance g_{AMPA} was modeled as the difference of two exponentials $g_{AMPA}(t) = g_{AMPA_max}(e^{-t/\tau 2} - e^{-t/\tau 1})$, with τ1 = 0.206 ms, τ2 = 0.26 ms (Franks et al., 2002). g_{AMPA_max} is the maximal conductance of the AMPARs (single channel conductance = 10 pS). The NMDA receptor current I_{NMDA} was calculated as $I_{NMDA} = g_{NMDA}G(V - E_{NMDA})$, where g_{NMDA} is

the total conductance, G describes the voltage-dependent Mg^{2+} block, and E_{NMDA} is the reversal potential of the NMDAR. The time course of the NMDA conductance was modeled as the sum of 3 exponentials:

$g_{NMDA}(t) = g_{NMDA_max}(0.88e^{-t/\tau 2} + 0.12e^{-t/\tau 3} - e^{-t/\tau 1})$ (Franks et al., 2002), with τ1 = 3.18 ms, τ2 = 57.14 ms, τ3 = 2000 ms (Hestrin et al., 1990; Spruston et al., 1995). g_{NMDA_max} is the maximal conductance of the NMDARs (single channel conductance = 45 pS). The equation describing the voltage-dependent Mg^{2+} block G (Jahr and Stevens, 1990) contains two empirically defined constants which we modified to fit our voltage-clamp experiments (Supplemental Fig. 1):

$$G = \frac{1}{1 + e^{-0.08V}(C/0.69)},$$ where C is the extracellular Mg^{2+} concentration (1 mM).

Approximately 10% of the current through NMDA channels is carried by calcium (Jahr and Stevens, 1993). Therefore, at a membrane potential of -65 mV, the calculated $P_{Ca,NMDA}$ is 10% of the total NMDA channel conductance (g_{NMDA}). The NMDA receptor current carried by Ca^{2+} was described as

$$I_{Ca_NMDA} = P_{Ca,NMDA}\eta(V - E_{NMDA,Ca})([Ca^{2+}]_i - [Ca^{2+}]_o)e^{(V-E_{NMDA,Ca})\mu} / (1 - e^{(V-E_{NMDA,Ca})\mu})G$$

$$\mu = -zF/RT, \qquad \eta = z^2F^2/RT$$

z is the valence of the ion, z = 2 for Ca^{2+}; F is Faraday's constant, 96,485 $Cmol^{-1}$; R is the gas constant, 8.314 $JK^{-1}mol^{-1}$; T is the absolute temperature in Kelvin, 307 K; $E_{NMDA,Ca}$ is the reversal potential of the NMDA channels for calcium (40 mV) (Schneggenburger et al., 1993; Spruston et al., 1995); $[Ca^{2+}]_o$ is the extracellular calcium concentration, $[Ca^{2+}]_o$ = 2 mM; and $[Ca^{2+}]_i$ is the intracellular calcium concentration, $[Ca^{2+}]_i$ = 50 nM (resting intracellular calcium concentration).

A voltage-gated calcium conductance was simulated using a Hodgkin-Huxley-like formalism adapted from Foehring et al. The current $I_{R\text{-type}}$ was described as $I_{R-type} = g_{R-type_max}m^3h(V - E_{Ca})$,

where m and h are the activation and inactivation variables, $g_{R\text{-type_max}}$ is the total VGCC conductance (single channel conductance = 17 pS). The reversal potential (E_{Ca}) was set to +10 mV to minimize the error (compared to the GHK current equation) in the range of membrane potentials where the channel was active (V_m = -40 mV to -10 mV). Activation and inactivation kinetics for $I_{R\text{-type}}$ were given by

$\tau_m \frac{dm(t)}{dt} = -m(t) + m_\infty$, $\tau_h \frac{dh(t)}{dt} = -h(t) + h_\infty$, with τ_m = 3.6 ms, τ_h = 200 ms, and corresponding steady-state equations (Foehring et al., 2000) for m_{inf}, h_{inf}

$m_\infty = \frac{1}{1+e^{-\frac{V-V_{m_half}}{k_m}}}$, $h\infty = \frac{1}{1+e^{-\frac{V-V_{h_half}}{k_h}}}$, with V_{m_half} = -14 mV, k_m = 6.7 mV^{-1}, V_{h_half} = -65 mV, k_m = -11.8 mV

The free parameters of our model were the densities of AMPARs, NMDARs, and R-type calcium channels in the spine head, and the diameter of the spine neck. We adjusted the current densities (Supplemental Table 1) to reproduce the calcium transient amplitude of the pharmacological experiments (Fig. 4) by systematic exploration of the parameter space (Supplemental Fig. 3).

Simulation of spine calcium transients

The increase of intracellular calcium, $[Ca^{2+}]_i$, is given by $\frac{d[Ca^{2+}]_i}{dt} = -\frac{I_{Ca}}{2FV}$,

where I_{Ca} is the net calcium current, F is Faraday's constant and V is the volume of the spine head. The net calcium current I_{Ca} is described by $I_{Ca} = I_{R-type} + I_{Ca_NMDA}$

$I_{R\text{-type}}$ is the calcium current through R-type VGCCs, and I_{Ca_NMDA} the calcium current through NMDARs. Since all our experiments were performed under conditions of high buffer concentration (600 μm fluo-5F), we used the simplifying assumption that all calcium ions that enter the spine were immediately bound by dye molecules. To model accumulation and diffusion of calcium-bound dye, we used a published NEURON mechanism that simulates radial diffusion between concentric shells

inside a compartment and longitudinal diffusion between adjacent compartments (Carnevale and Hines, 2006). The diffusion mechanism was inserted to the spine, spine neck, and connected dendritic compartments (D = 0.23 µm^2/ms, diffusion coefficient of fluo5F-Ca^{2+} in cytoplasm). For display purposes, we averaged the concentration of calcium-bound dye across all sub-compartments of the spine head. Without introduction of any additional free parameters (Ca^{2+} removal mechanisms), the experimentally measured time course of the fluorescence transients under different conditions was well captured by the simulation (Fig. 4A and C).

Estimation of spine neck resistance

In our simulations, the strong effect of AMPA receptor block on calcium signal amplitude was only reproduced with Rneck ~1.2 GΩ. Is such a high neck resistance consistent with the decay time constants measured in the calcium imaging experiments? The average fluorescence decay time constant of all spines with a clear calcium response at resting membrane potential (excluding 'calcium-silent' spines) was τ = 0.9 s (Fig. 2D). Based on these measurements, we calculated the spine neck length to cross section ratio (L/A) according to the equation (Bloodgood and Sabatini, 2005)

$$L/A = \tau \cdot D / V = 900\ [\text{ms}] \cdot 0.1\ [\mu\text{m}^2/\text{ms}] / 0.11\ [\mu\text{m}^3] = 818\ [\mu\text{m}^{-1}]\ ,$$

where D is the diffusion coefficient of fluo-5F in cytoplasm (100 µm^2/s) (Michailova et al., 2002) and V is the average spine volume of our sample (0.11 µm^3).

We estimated the resistance of the spine neck according to the cable equation

$$R_{\text{neck}} = R_{\text{i}} \cdot L / A = 150 \cdot 10^4\ [\Omega\ \mu\text{m}] \cdot 818\ [\mu\text{m}^{-1}] = 1.2 \cdot 10^9\ [\Omega]$$

assuming an internal resitivity Ri = 150 Ωcm.

Thus, the spine neck resistance that produced realistic interactions between AMPA, NMDA and R-Type channels in the NEURON simulation (1.2 GΩ, Fig. 4C) is also consistent with the decay time constants we found experimentally (Fig. 2D).

SUPPLEMENTAL TABLES

Supplemental Table 1: Number of channels used in simulation

	AMPA receptor	**NMDA receptor**	**R-type VGCC**
# of channels present in spine	240	110	230
maximum # of channels open during EPSP	240	18	9

Supplemental Table 2: Electrotonic attenuation of EPSC and EPSP (1.2 GΩ spine neck)

	EPSC amplitude (Voltage clamp)	**EPSP amplitude (Current clamp)**
spine head	41 pA	54.8 mV
spine base	n/a	22.2 mV
soma	24 pA	1.1 mV

SUPPLEMENTAL REFERENCES

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