SI Methods

Neuronal Culture Preparation and Stimulation. Dissociated hippocampal cell culture was prepared as described in ref. 1. Briefly, rats were killed on postnatal day 0-1, hippocamppi dissected, dissociated and plated at 25,000 cells per coverslip on 18 mm coverslips coated with a monolayer of purified hippocampal astrocytes. Cultures were grown for 7-8 DIV, transfected with SuperGluSnFR via calcium phosphate precipitation (2), allowed to mature to 14-17 DIV and assayed. Highly expressing neurons with clear plasma membrane expression were selected for optical measurements. Great care was taken to minimize light exposure and bleaching throughout the experiment. Neurons were imaged with a mercury arc lamp with 0.33-1.5% neutral density transmission and 420/20 excitation filters. The emission beam was split with a DualView (Optical Insights) with a CFP/YFP filter set (OI-05-EX), recorded at 30 fps by a Hamamatsu EB-CCD camera (C7190-53), digitally recombined and ratioed with SimplePCI (CImaging). High-speed single AP imaging was performed with a Cascade 128+ (Roper) camera in 3 × 3 binning mode and Metafluor 6.1 (UIC). Spatially resolved ratio changes were calculated by using custom MATLAB processing routines.

Field stimulation was delivered by a Grass stimulator (SD9) to a custom chamber, similar to Warner Instruments RC-21BRFS, with custom control software. Stimulator settings were 0.3 ms, 70-80V biphasic pulse per action potential. All neuronal manipulations were done at room temperature in Ringer's solution with 2 mM [Ca²⁺], 1.3 mM [Mg²⁺], supplemented with 25 μ M NBQX and 50 μ M APV to block recurrent excitation from glutamate perfusion or release. For uptake regulation experiments (Fig. 5 *a* and *b*), four traces of each 1AP and 2 traces of each 10AP condition were obtained in 8 neurons, converted to glutamate concentration, then averaged. Glutamate decays were poorly fit by single exponentials, so times to half decay are reported. To block active reuptake, 100 μ M TBOA was selectively added to the bath. To simplify diffusion modeling, stimulations were performed in a still bath rather than under continuous perfusion.

Sensor Calibration. Because GluSnFRs are ratiometric indicators, backgroundsubtracted responses should be independent of indicator concentration. However, when expressed on the surface of mammalian cells, baseline SuperGluSnFR FRET ratios and ΔR_{max} are variable (Fig. 2e), depending on cell type, reporter expression level, autofluorescence background and reporter bleaching. Initially, optical glutamate titration curves were generated by bath changes of a broad range of [glutamate] in Ringer's solution. Absolute CFP/YFP emission ratio changes were normalized to the maximum change. A variable-slope dose-response fit was performed on the average normalized change to find the apparent K_d for glutamate. To make quantitative measurements of [glutamate] in neuronal experiments, each cell's ΔR_{max} was determined by calibration with two known glutamate concentrations, 0 and 100 µM, with synaptic responses normalized to this ΔR_{max} .

Pixel intensities of ROIs in the CFP and YFP emission were averaged, background subtracted, then ratioed. Typical ratio bleaching curves for the light intensities we used had a fast exponential drop in the first several hundred milliseconds of illumination followed by a near linear, partially dark-reversible bleaching of ≈ 0.5 to 2% absolute ratio over the next several seconds. Over multiple illuminations, the slope of these two components evolved significantly. Thus, a biexponential fit of a single canonical bleach curve was inadequate to reliably characterize the bleach rate, whereas multiple interleaved bleaching curves caused an unacceptable loss of signal by the end of each experiment. Therefore, after discarding the initial fast component, we scaled the ratio by a linear fit of the prestimulus bleach rate for each trace. This method provided reliable correction for the first two seconds after each stimulus, with increasing divergence at longer time points.

To correct for intertrial changes in sensor bleach levels and autofluorescence, prestimulus ratios were multiplicatively scaled to the [glutamate] free calibration ratio for that cell. After these corrections, average extracellular [glutamate] (glu_{ex}) was calculated with the formula $K_d^*((R_{\text{free}} - R)/(R - R_{\text{sat}}))^*(\text{CFP}_{\text{free}}/\text{CFP}_{\text{sat}})$, where K_d is 2.5 μ M, R equals the

current YFP/CFP emission, R_{free} and R_{sat} are YFP/CFP emission in zero and 100 μ M glutamate, and CFP_{free} and CFP_{sat} are CFP emission in zero and 100 μ M glutamate (3).

Determination of Background Glutamate Concentration. In neuronal cultures without TBOA in the bath solution, glutamate titrations of SuperGluSnFR gave a curve with apparent K_d of 8.9 µM and a Hill coefficient of 1.5 (Fig. 2*a*). We formulated two hypotheses for the discrepancy between this curve and the HEK293/HeLa titration curves. First, micromolar levels of background glutamate may exist near the surface of neurons in equilibrium between spontaneous glutamate release, reuptake and diffusion. *In vivo* microdialysis has estimated background glutamate levels in the extracellular fluid of the cerebellum at 2.0 µM, although these levels may be overestimates due to probe-induced tissue damage (4). More recent estimates in slice place the extracellular glutamate would raise the response floor, increasing the apparent sensor K_d and Hill coefficient (SI Fig. 7*a*). Alternatively, the astrocyte uptake capacity may outpace the diffusion of glutamate from the bath onto the culture surface, causing surface depletion of neurotransmitter after glutamate application.

To test the first hypothesis, we rapidly perfused Ringer's with 1 μ M glutamate directly onto SuperGluSnFR expressing neurons during continuous FRET ratio monitoring. This induced a rapid increase in CFP/YFP ratio, indicating the pipette location was appropriate to induce GluSnFR responses and also providing an upper bound to the background [glutamate]. Perfusion of glutamate-free Ringer's from the same unmoved pipette had no effect on the CFP/YFP ratio, indicating background glutamate levels were below the level of detection of SuperGluSnFR (SI Fig. 7*b*).

Using a numerical model of glutamate diffusion and uptake we checked whether local depletion by transporter uptake was sufficient to explain the shifted titration curve. The concentration timecourse after bath exchange of varying levels of glutamate was calculated assuming no significant spontaneous release from neurons and astrocytes. Model equations were as in SI Scheme 1, with glutamate added homogenously

throughout the bath at t = 0. After glutamate applications, the model showed significant depletion of glutamate near the coverslip surface (SI Fig. 7*c*) that was proportionally greater for smaller bath concentrations (SI Fig. 7*d*). By lowering the uptake rate (k_2) or transporter concentration (U_{max}) such that the total maximum uptake rate was 40,000 molecules s⁻¹/µm² coverslip, we were able to qualitatively fit the TBOA-free neuronal titration curve with locally depleted glutamate levels at the 30 second time point (SI Fig. 7*a*, green). This indicated transporters were sufficient to rapidly deplete surface glutamate levels after bath application.

Glutamate Dynamics and Receptor Modeling. Electrically evoked glutamate release was modeled as an instantaneous injection of homogeneously distributed glutamate (1.5 μ M, equivalent to 0.192 vesicles/ μ m3 at 4700 glutamates/vesicle) in the neuronal plane for the first AP of each stimulation. For each successive AP, a decreasing amount of glutamate was injected, estimated by a double exponential association fit of synaptopHluorin fluorescence increases for 30Hz and 15Hz 10AP field stimulations (data not shown). The tenth action potential injected 43% and 47% of the first for the respective 30Hz and 15Hz cases. Additional asynchronous release was not considered. To account for the interlaced filtering effects of the Hamamatsu camera, model glutamate concentration was integrated with a 33.3 ms time window and averaged with a 33.3 ms delayed duplicate trace.

After release, glutamate diffused through the neuropil region, became transiently bound by SuperGluSnFR, glutamate transporters or NMDA receptors, became bound and internalized by transporters, or diffused into the bath (SI Scheme 1). Glutamate diffusion was modeled as 1-dimensional diffusion from the release plane using the central difference approximation for space discritization and Euler's method for time discritization. Step intervals were 3.3 μ s and 250 nm and total simulation duration was 2 seconds. Shorter intervals did not improve macroscopic fit properties. GluSnFR, transporter and NMDAR location was assumed to be homogeneous within the neuronal plane. Diffusion rate ($D = 0.76 \,\mu$ m²/ms), and reuptake and GluSnFR binding equations were adapted from Barbour and Hausser (6). Because the culture neuropil was heterogeneous with unknown geometries and bath exposure, tortuosity and extracellular volume reduction parameters were omitted.

Glutamate transporters were considered as a single species with rate constants ($k_1 = 10^7$ $M^{-1}s^{-1}$, $k_{-1} = 86 s^{-1}$, $k_2 = 14 s^{-1}$) (7). Maximum free binding concentration ($U_{max} = 10 \mu M$) was set at somewhat less than literature estimates for hippocampal slice (8). With TBOA in the bath, U_{max} was set to zero. For the NMDA receptors, off rates and K_d values were derived from the equation $K_d = (\sqrt{2}-1) \text{ EC}_{50}$, assuming the EC₅₀ (EC_{50:2A} = 1.7 μ M, $EC_{50:2B} = 0.8 \mu M$, $EC_{50:2D} = 0.4 \mu M$) (9, 10) reflects two independent glutamate binding events ($k_1 = 5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1:2A} = 3.52 \text{ s}^{-1}$, $k_{-1:2B} = 1.66 \text{ s}_{-1}$, $k_{-1:2D} = 0.83 \text{ s}^{-1}$). Total NMDAR binding sites were 300 nM equally distributed between NR2A, 2B and 2D subtypes. We used the lower bound rate constants of SuperGluSnFR ($k_1 = 3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = 75 \text{ s}^{-1}$). GluSnFR concentration was estimated at $\approx 200 \text{ molecules}/\mu\text{m}^2$ coverslip (eq. to 127 nM homogeneously distributed in the neuropil) by calibrating pixel intensities to thin optical chambers filled with known concentrations of purified SuperGluSnFR (data not shown). Vesicle release rates were roughly estimated with synaptopHluorin imaging (data not shown) and adjusted to fit the rising phase of the average 1AP, 10AP-15Hz and 10AP-30Hz in TBOA GluSnFR traces. The model captured the essential timecourse of spillover rise and decay, although the degree of frequency-dependent facilitation in the active uptake case was consistently lower in the model (Fig. 5c). This discrepancy was perhaps due to the dramatic simplification of the spatial distribution of release and transporters. No significant glutamate accumulated in the far end of the model chamber within 2 seconds indicating use of a closed-boundary condition model was accurate for these timescales (SI Fig. 8b).

Although the glutamate release kinetics are constrained by the TBOA records, glutamate transporter kinetic estimates vary significantly (11, 12), and our estimate of the concentration of transporters in the culture system is imprecise. Therefore, we perturbed the model to assess the system's parametric sensitivity. Increasing [SuperGluSnFR] from 200 molecules/ μ m² coverslip to 1500 molecules/ μ m² (1 μ M) had a negligible effect on the modeled glutamate transients, indicating buffering by the sensor had little impact on

glutamate transients on the timescale of tens of milliseconds (SI Fig. 8*c*). A threefold increase in either transporter k_2 or U_{max} caused a precipitous drop in spillover glutamate during stimulation, whereas decreasing either caused a reciprocal increase in spillover (SI Fig. 8 *d* and *e*). Therefore, the model was most sensitive to the total internalization capacity of glutamate by transporters. Our data do confirm that published estimates in a simple uptake model can reproduce our quantitative optical measurements of spillover glutamate dynamics. Furthermore, it suggests that the magnitude of glutamate spillover is highly sensitive to total uptake capacity.

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Fig. 6. (*a*) Emission spectrum of soluble GluSnFR_{0N0C} before and after digestion with trypsin. Excitation of 420 nm. (*b*) Partial reversal of glutamate binding by conversion of glutamate to α -ketoglutarate. Glutamate-free (black), plus 333 nM [glutamate] (*red*), after 10 min with glutamate-pyruvate transaminase and 10 mM pyruvate (blue). (*c*) Responses of GluSnFR to 2.5 μ M glutamate in the presence of 300 μ M NMDA, 100 μ M AMPA, 100 μ M KA, 25 μ M ACPD and 250 μ M MCPG normalized to the response to 2.5 μ M glutamate alone (*n* = 4 fields per condition).



Fig. 7. (*a*) Glutamate titration curves of HEK/HeLa cells in Ringer's (black squares, dashed line), neurons in Ringer's (black circles, solid line), neurons adjusted with 2 μ M background glutamate (yellow squares, solid line), modeled apparent titration curves at 30 s after glutamate bath exchange (green triangle, dotted line), and neurons in Ringer's plus 100 μ M TBOA (diamonds, red). (*b*) Perfusion of GluSnFR neuron with 1 μ M [glutamate] (red), glutamate-free Ringer's (blue), vs. no perfusion (black). (*c*) Modeled glutamate depletion after bath application of 10 μ M glutamate. Depletion is rapid and significant within the first 100 μ M from the neuronal plane. (*d*) Glutamate depletion at the coverslip surface for various initial concentrations of applied glutamate. Relative depletion is greater for smaller initial [glutamate] because of the decreased diffusive flux into the neuron-astrocyte plane.



Fig. 8. Model of glutamate release, diffusion and reuptake. (*a*) Predicted SuperGluSnFR response to a homogeneously injected transient of 3 μ M [glutamate] (gray) with k_{on} and k_{off} of 9 × 10⁷ M⁻¹ s⁻¹ and 225 s⁻¹ (green), 3 × 10⁷ M⁻¹ s⁻¹ and 75 s⁻¹ (red), and 1 × 10⁷ M⁻¹ s⁻¹ and 25 s⁻¹ (blue). Measured SuperGluSnFR response (black circles). (*b*) Spatio-temporal profile of model glutamate after 10 AP 30 Hz stimulation in Ringer's with TBOA. Distance is measured from coverslip surface. (*c*) Increasing [GluSnFR] from 200 (red) to 1,500 (blue) molecules per square micrometer coverslip had no effect on modeled GluSnFR response. (*d*) Varying the glutamate transporter internalization rate between 42 (green), 14 (red) and 4.3 (blue) molecules s⁻¹ had a profound effect on spillover glutamate. (*e*) Varying effective transporter concentration between 3.3 μ M (blue), 10 μ M (red) and 30 μ M (green) had a similar effect as *d*.

SI Scheme 1. Model Kinetic Scheme.

$$\begin{aligned} \frac{\partial [glu]}{\partial t} &= D \left\{ \frac{\partial^2 [glu]}{\partial x^2} \right\} + \left[k_{-1} (U_{max} - U) - k_1 U[glu] \right] + \frac{dG}{dt} + \frac{dN_{2A}}{dt} + \frac{dN_{2B}}{dt} + \frac{dN_{2D}}{dt} \\ \frac{dU}{dt} &= (k_{-1} + k_2) (U_{max} - U) - k_1 U[glu] \\ \frac{dG}{dt} &= k_{-1} (G_{max} - G) - k_1 G[glu] \\ \frac{dN_{2A}}{dt} &= k_{-1} (N_{2A:max} - N_{2A}) - k_1 N_{2A} [glu] \\ \frac{dN_{2B}}{dt} &= k_{-1} (N_{2B:max} - N_{2B}) - k_1 N_{2B} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_1 N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_1 N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_1 N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_1 N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_1 N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_1 N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_1 N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_1 N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D:max} - N_{2D}) - k_{1} N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D} [glu] \\ \frac{dN_{2D}}{dt} &= k_{-1} (N_{2D} [glu] \\$$

State transitions and differential equations governing the glutamate diffusion, binding, and uptake model. Glutamate is modeled as one-dimensional diffusion from a homogeneous thin source. In the equations, *glu* is the extracellular glutamate; *glu_{in}* is intracellular glutamate; *D* is the diffusion coefficient; *t* is time; *x* is distance, *U*, *G*, *N*_{2A}, *N*_{2B}, and *N*_{2D} are the unliganded concentrations of glutamate transporters, GluSnFR, NMDA NR2A, 2B, and 2D receptors; U_{max} , G_{max} , $N_{2A:max}$, $N_{2B:max}$, and $N_{2D:max}$ are the total concentration of transporters, GluSnFR, NR2A, NR2B, and NR2D receptors. Rate constants k_1 , k_{-1} , and k_2 are different for each reaction species. All components are homogeneously distributed in the neuronal plane.