Stability, affinity, and chromatic variants of the glutamate sensor iGluSnFR

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Single-wavelength fluorescent reporters allow visualization of specific neurotransmitters with high spatial and temporal resolution. We report variants of intensity-based glutamate-sensing fluorescent reporter (iGluSnFR) that are functionally brighter; detect submicromolar to millimolar amounts of glutamate; and have blue, cyan, green, or yellow emission profiles. These variants could be imaged in vivo in cases where original iGluSnFR was too dim, resolved glutamate transients in dendritic spines and axonal boutons, and allowed imaging at kilohertz rates.

The glutamate sensor iGluSnFR has become an invaluable tool for studying glutamate dynamics in diverse systems, including retina, mouse olfactory bulb, and cat visual cortex. iGluSnFR also allows mesoscale ‘functional connectomic’ mapping and mechanistic studies of excitatory vesicle fusion, synaptic spillover, cortical spreading depression, and Huntington’s disease. However, iGluSnFR has limitations: the reporter is too slow to follow fast synaptic transients, and too insensitive to detect sparse release. Here we describe variants that are functionally brighter (as a result of increased expression); have higher or lower affinity (because of slower or faster off-rates, respectively); and fluoresce blue, cyan, green, or yellow.

We found that replacement of circularly permuted eGFP with circularly permuted superfolder GFP (SF-iGluSnFR) increased the thermodynamic stability and soluble-protein expression level of iGluSnFR in bacteria, without changing the two-photon cross-section or the excitation, emission, and absorption spectra (Supplementary Fig. 1).

SF-iGluSnFR was expressed robustly in mouse somatosensory cortex, and was bright enough for typical resonance-scanner-based two-photon imaging in vivo (<20 mW at the sample; Supplementary Fig. 2a,b), whereas original iGluSnFR was unobservable. When we increased the laser power to make original iGluSnFR observable (Supplementary Fig. 2c,d), we noted that partially bleached SF-iGluSnFR was still brighter than unbleached original iGluSnFR (Supplementary Fig. 2e,f). Furthermore, continuous imaging of a single spine expressing SF-iGluSnFR in the barrel cortex showed negligible bleaching during a 7-min experiment (Supplementary Fig. 2g). In mouse retina, where original iGluSnFR is expressed well, SF-iGluSnFR was much more photostable (Supplementary Fig. 3).

Although the affinity of iGluSnFR is adequate for some in vivo applications, faster off-rate variants are needed to resolve fast glutamate transients associated with local glutamate release. Mutation of S72 in the glutamate-binding pocket to alanine resulted in a solubility protein (SF-iGluSnFR.S72A) with a slower on-rate, faster off-rate, and 200 µM affinity for glutamate (Supplementary Fig. 4a,b). The affinity of Venus-flytrap-like binding proteins can be altered without compromising the stereochemical integrity of the ligand-binding site through mutations in the ‘hinge’ region and allosteric shifting of open and ligand-free to closed and ligand-bound equilibrium1. We screened a saturating A184X library of SF-iGluSnFR (A184V in original iGluSnFR). Reversion to alanine or other small amino acids increased affinity (A184A had a low ∆F/ΔF, whereas larger side chains decreased affinity (Supplementary Fig. 4c). A184S had a slower off-rate and showed an increase in affinity from 40 µM (A184V) to 7 µM (A184S; Supplementary Fig. 4a,b).

We recloned these SF-iGluSnFR variants (S72A (low affinity), A184V (original), and A184S (high affinity)) into an adenovirus vector containing an IgG secretion signal and a PDGFR transmembrane domain. Expression of the membrane-tethered version in cultured rat hippocampal neurons increased its affinity by an order of magnitude (Supplementary Fig. 5a), as was observed with the original sensor7. Whole-field electrical stimulation (50 Hz) of neuronal cultures expressing SF-iGluSnFR variants increased fluorescence, with decay rates that paralleled the in vitro kinetics; all variants decayed faster than the calcium sensor GCaMP6f (Supplementary Fig. 5b). A recently published iGluSnFR.S72T variant also has a fast off-rate (ref. 14), but SF-iGluSnFR.S72T showed a lower ∆F/ΔF than S72A in our hands.

In vivo, the high-affinity SF-iGluSnFR.A184S variant improved detection of stimulus-evoked glutamate release in ferret visual cortical neuropil in response to drifting gratings (Fig. 1). In neuropil, peak amplitudes reached 30% ∆F/ΔF for SF-iGluSnFR.A184S (Fig. 1a–c) but only 5% ∆F/ΔF for SF-iGluSnFR.A184V (Supplementary Fig. 6), and were not apparent with original iGluSnFR (data not shown). We also saw detectable, but lower-magnitude, changes in fluorescence on cell bodies with SF-iGluSnFR.A184S (Fig. 1a–c). The greater ∆F/ΔF of SF-iGluSnFR.A184S allowed extraction of robust orientation tuning curves in regions of sparse labeling (Fig. 1d, Supplementary Fig. 7). We
were also able to resolve orientation-selective responses in individual dendritic spines, with some spillover onto nearby dendrites (Fig. 1e,f).

Some individual spines showed robust orientation selectivity (Fig. 1g), whereas other spines on the same dendrite exhibited little or no visually driven activity (Supplementary Fig. 7c). The response magnitude at spines was stable over multiple stimulus presentations, with no appreciable loss in fluorescence intensity or $\Delta F/F$ (Supplementary Fig. 8). We found that response amplitudes across individual trials were consistently greater in the A184S variant than in the A184V variant (Supplementary Fig. 6) when we considered all stimulus-evoked responses (A184S, median $\Delta F/F = 16\%$, $n = 72$ spines; A184V, median $\Delta F/F = 9\%$, $n = 22$ spines; $P < 0.0001$, Wilcoxon rank-sum test) or only preferred stimuli (A184S, median $\Delta F/F = 27\%$, $n = 72$; A184V, median $\Delta F/F = 14\%$, $n = 22$; $P < 0.0001$; Supplementary Fig. 7b). Single-trial responses from individual spines showed clear orientation selectivity and displayed robust glutamate events with high signal-to-noise ratio (SNR; Supplementary Fig. 7d,e, Supplementary Video 1).

The faster off-rate of SF-iGluSnFR.S72A enabled more accurate quantification of quantal synaptic responses at high stimulation frequencies than is possible with SF-iGluSnFR.A184V or original iGluSnFR (Fig. 2a,b, Supplementary Fig. 9). We modulated transmitter release and its temporal depression by changing the extracellular calcium concentration. SF-iGluSnFR.A184V and original iGluSnFR could not distinguish the differences in the temporal pattern of transmitter release between 1 mM and 3.5 mM $\text{Ca}^{2+}$ during a brief stimulus train (20 Hz). In contrast, S72A reported the conversion from facilitation of individual responses during the stimulus train to a strong depression after a switch from low to high extracellular $\text{Ca}^{2+}$ concentration (Fig. 2c). This makes SF-iGluSnFR.S72A more useful for assessing short-term synaptic plasticity. S72A also enhanced the spatial resolution of glutamate release compared with that achieved with A184V (Supplementary Fig. 10).

We next examined whether SF-iGluSnFR could be used as an alternative to GCaMP6f to monitor the presynaptic activity of granule cell axons within mouse cerebellar brain slices. Axons of granule cells bifurcate in the molecular layer of the cerebellar cortex to form parallel fibers (PFs; Fig. 2d), which can be reliably stimulated by local extracellular stimuli at high frequencies than is possible with SF-iGluSnFR.A184V or original iGluSnFR (Fig. 2a). As the average of three to ten fluorescence traces from a single bouton would have noise similar to that of three to ten boutons averaged in one trial (Fig. 2e), we suggest that presynaptic APs could be inferred from SF-iGluSnFR responses. We analyzed population averages to demonstrate that SF-iGluSnFR variants were sufficiently fast to report single events even in response to 100 Hz stimulation, in contrast to GCaMP6f (Fig. 2f, Supplementary Fig. 12c). Therefore, provided that a sufficient number of boutons from the same axon could be recorded simultaneously, SF-iGluSnFR, unlike GCaMP6f, could allow the detection of PF firing frequencies typical of the high instantaneous firing rates ($> 100$ Hz) of cerebellar granule cells.

The introduction of chromophore mutations from GFP variants Azurite, mTurquoise2, and Venus to SF-iGluSnFR led to blue, cyan, and yellow versions, respectively (Supplementary Fig. 13). For SF-Azurite-iGluSnFR and SF-mTurquoise2-iGluSnFR, reoptimization was required in order to increase $\Delta F/F$ (Supplementary Fig. 14). SF-Venus-iGluSnFR was a straightforward modular replacement with similar affinity and maximum fluorescence response as SF-iGluSnFR, but with red-shifted excitation and emission...
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The high output power of fiber lasers allows for simultaneous excitation of multiple APs. Results were repeated in multiple boutons:

- We recorded single-spine fluorescence transients from sparsely populated areas.
- The propagation of fluorescence through the sample was consistent with diffusion of glutamate.
- In mouse visual cortex, we observed strong orientation-selective responses in single trials.

The SF-iGluSnFR variants described here could increase the power of genetically encoded glutamate imaging. Affinity variants with altered kinetics broaden the dynamic and concentration ranges of observable glutamate transients. Chromatic mutants allow fast imaging with lower-cost lasers, and open a path to multicolor imaging in conjunction with other probes. In all applications, improvements in membrane targeting and photostability will increase precision, especially in complex tissues such as brain neuropil, where synapses outnumber neurons by a factor of thousands. We expect that together, these variants will substantially increase the potential of glutamate imaging to monitor brain function.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41592-018-0171-3.
Fig. 3 | Utility of SF-Venus-iGluSnFR in cultured neurons and in vivo. a–c, High-speed (1,016-Hz frame rate) two-photon imaging of a cultured neuron expressing SF-Venus-iGluSnFR.A184V. a, RuBi-glutamate was uncaged for 10 ms at each of two 5-µm spots (arrowheads) on two adjacent dendrites (Supplementary Video 2). Short saturation denotes the glutamate transient amplitude, and color hue denotes response timing (see key). Gray tones indicate no evoked response. The yellow line indicates the axis of the kymograph shown in c. Representative results from 1 of 4 trials. b, Traces recorded at the centers of the uncaging locations shown in a. c, Top: kymograph showing the response amplitude over time along the dendrite surrounding uncaging location 1 in a. Traces are approximate maximum-likelihood solutions for a spatially multiplexed recording acquired with a high-power 1,030-nm fiber laser. These recordings show a single trial without averaging. Bottom: simulation (Methods) of diffusion of glutamate at the surface of a planar coverslip surrounded by 3D solution, after uncaging in a 5-µm spot with a 0.8-NA beam at time 0. The intensity scale is log-transformed. d–f, Recording of visually evoked spine transients in isolated SF-Venus-iGluSnFR.A184S-labeled dendrites in mouse visual cortex, imaged at 1,030-nm excitation. See also Supplementary Video 3. d, Motion-aligned average image. e, Mean responses (top) and tuning curves (bottom) of ROIs indicated in d, for 20 trials of each of the 8 oriented moving grating stimuli. Dark lines/markers denote means; shaded regions/error bars denote s.e.m. The black bars at the top denote the stimulus period. f, Pixel intensity of ROI 1 for eight consecutive stimulus presentations of different directions, illustrating detectable responses in single trials. Colored bars denote stimuli as in e. Panels d–f show example results representative of 7 mice.

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Author contributions
J.S.M., L.L.L., A.N.T., and E.C. carried out protein engineering; B.S., D.E.W., and D.F. conducted experiments on ferret visual cortex; J.A.M., S.S.-H.W., A.W.H., and D.A.D. conducted cerebellum experiments; V.J.D. and B.G.B. conducted retina experiments; and J.S.M. and L.L.L. wrote the manuscript.

Competing interests
J.S.M. and L.L.L. are named on US patent 9719992, which pertains to original iGluSnFR.

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Methods

In vivo assessment of iGluSnFR brightness and photostability in apical dendrites in mouse somatosensory cortex. All procedures were approved by the Janischel Institutioanl Animal Care and Use Committee and Institutional Biosafety Committee (UCSF). C57BL/6J mice were purchased from the Jackson Laboratory and group-housed in the Janischel animal facility. Mice (male and female) were injected at 8 weeks of age with AAV2/1.hSynapsin1.iGluSnFR.A184S or SF-ΔiGluSnFR.A184S, at identical titers (1 × 1013 genomic copies (GC)/ml), volumes (20 nl), and locations (3 mm lateral to midline, 1.4 mm caudal to bregma, and 0.3 mm below the cortical surface). After viral injection, a craniotomy (3-mm diameter) was made over the injection site, and the removed portion of the skull was replaced with a z 1.5 Schott glass fixed in place with dental acrylic (Lync Dental Manufacturing), which also secured a titanium head bar to the skull for head-mounting during imaging experiments.

In vivo two-photon imaging experiments were performed while the mice were in a state of quiet wakefulness, after they had been habituated to head fixation over the prior 2–3 d. Periodic water rewards were given to keep animals hydrated and passive. For comparisons of intensity and bleaching, we used a custom two-photon microscope emitting 960-nm light from a Coherent Chameleon ultrafast laser. All experiments were performed with a 23x/1.05-NA (numerical aperture) Olympus objective immersed in water. Imaging acquisition was performed with ScanImage software and analyzed post hoc with ImageJ. All images were analyzed at a variety of speeds, zooms, and powers to assess the effects of pulse energy and dwell time on bleaching and intensity. Images at each setting were acquired for 5 s.

For analysis, images were averaged and thresholded to create a signal (above threshold) and background (below threshold) for each pixel. Signals were then subtracted, and SNR was calculated from these as \[ \text{SNR} = \frac{\text{signal} - \text{background}}{\text{s.d. of background}} \].

Mouse retinal explant imaging for assessment of iGluSnFR brightness and photostability. Retinas were prepared as previously described. iGluSnFR was expressed in retinal neurons after injection of 0.8–1.0 µl of AAV2/1.hSynapsin1 virus (1 × 1013 GC/ml) into the vitreous humor of adult mouse eyes using a custom-designed syringe (Borghuis Instruments, New Haven, CT). After 17–21 d, eyes were dissected in oxygenated Ames medium (93% O2, 5% CO2; Sigma–Aldrich) under infrared illumination (OWL Night Vision Scopes, third generation; B. E. Meyer), and each retina was removed from the sclera and mounted in a perspex ring (9 mm diameter) made of vacuum formable acrylic, which also secured a titanium head bar to the skull for head-pressuring during in vitro imaging experiments.

Viral transduction and image acquisition. Viral transduction and image acquisition were performed as previously described1. Viral transduction and image acquisition were performed as previously described1. After 4 weeks, ferrets were anesthetized with 30 mg/kg ketamine and 1–3% isoflurane. Atropine (0.2 mg/kg) and bupivacaine were administered subcutaneously. Animals were kept at 37–38 °C, artificially respirated, and given intravenous fluids. Isoflurane (1–2%) was used throughout the surgical procedure to maintain a surgical plane of anesthesia. Electrocardiograms, endtidal CO2, external temperature, and internal temperature were continuously monitored. A custom titanium head plate was implanted on the skull at the viral injection site, and the dura was retracted to reveal the cortex. A custom insert with a single 5-mm coverslip (0.17-mm or 0.7-mm thickness) was placed onto the brain to gently compress the underlying cortex and dampen biological motion during imaging. The cranial window was hermetically sealed using a stainless steel retaining ring and Vetbond. Tropicanide ophthalmic solution and phenylephrine hydrochloride ophthalmic solution were applied and contact lenses were inserted into both eyes.

Upon completion of the surgical procedure, isoflurane was gradually reduced and pancuronium (2 mg/kg/h) was delivered intravenously to immobilize the animal. The animal was placed under the microscope 25 cm from the stimulus monitor, with the monitor subtending 130° in azimuth and 74° in elevation. Imaging was carried out with a Bergamo II (Thorlabs) running ScanImage 5 or ScanImage 2015 (Vidrio Technologies) with dispersion-compensated 950-nm excitation provided by an Insight DS+ (Spectraphysics). Average excitation power after the exit pupil of the objective (16x; CF175; Nikon Instruments) ranged from 25 to 40 mW. Two-photon frame triggers from ScanImage were synchronized with stimulus information using Spike2 (CED). Visual stimuli were generated with PsychoPy2. Full-field drifting square-wave gratings (16 directions, 100% contrast, 0.1 cycles/degree, 4 cycles/s, 3-s stimulus period followed by 2–3 s interstimulus interval, plus a blank) were presented to the contralateral eye in a pseudorandom sequence for eight to ten trials.

Images were corrected for photomechanical motion via a correction-based approach (MATLAB). Regions of interests (ROIs) were drawn in ImageJ. Fluorescence time courses were computed as the mean of all pixels within the ROI at each time point and were extracted with Miji. Fluorescence time courses were then synchronized with stimulus information, and visually evoked responses were computed as changes in fluorescence relative to the baseline fluorescence. Peak ΔF/F responses for field ROIs and dendritic spines were computed via Fourier analysis to calculate mean and modulation amplitudes for each stimulus presentation, which were summed together.

For identification of spine SF-iGluSnFR events, ΔF/F traces during individual stimulus trials were smoothed with an exponentially weighted moving average filter (MATLAB), and peaks of possible ΔF/F events were identified. Identified spine event amplitudes were compared to the s.d. of baseline spine fluorescence values.

Mouse neuronal culture analysis. Primary hippocampal neuron cultures. Primary hippocampal neuron cultures were prepared from embryonic mice (embryonic day 16) as described previously1. Hippocampi were rinsed three to five times in Hank’s Balanced Salt Solution (HBSS; Life Technologies) and digested with trypsin (25 mg/ml; Life Technologies) for 20 min at 37 °C followed by DNase I (1 mg/ml; Roche). Subsequently, the tissue was triturated using cannulas (three times at 0.9 mm × 40 mm; three times at 0.45 mm × 23 mm) and the solution was passed through a BD cell strainer (70 µm). Fractions of the viral particles were prepared via 4–10 ml of basal medium Eagle (Life Technologies) supplemented with 0.5% glucose (Sigma–Aldrich), 10% FCS, 2% B-27, and 0.5 µM l-glutamine (all Life Technologies) to collect all cells. After counting, the cells were plated on coverslips in a 24-well cell culture plate at a density of 70,000 cells per 24-well plate and cultured in a humidified incubator at 37 °C and 5% CO2.

Viral vector production. Recombinant AAV2/1 genomes were generated by large-scale triplenfection of HEK293 cells as described previously1. The adenovirus associated virus (AAV) viral plasmid coding for SF-ΔiGluSnFR.S72A or SF-ΔiGluSnFR.A184V, helper plasmids encoding rep and cap genes (pBV1 and pH21), and adeno viral helper pfA6 (Stratagene) were transfected via calcium phosphate transfection method. Cells were harvested 72 h after transfection. Purify the virus, we lysed cell pellets in the presence of 0.5% sodium deoxycholate (Sigma) and 50 units/ml Benzonase endonuclease (Sigma). rAAV viral particles were purified from the cell lysate by EriTrap heparin HP column purification (GE Healthcare) and then concentrated. Ultrafiltration centrifugal filters (Millipore) until a final stock volume of 500 µl was reached.

Viral transduction and image acquisition. Primary hippocampal neuron cultures were transduced with AAV2/1.hSynapsin1.SF-ΔGluSnFR.R5T2A or AAV2/1.hSynapsin1.SF-ΔGluSnFR.A184V, at 1013 GC/ml in vitro (DIV) using a DIV 9. A low-frequency field stimulation (1 ms, 20 mA; platinum bar electrodes) was applied to recruit a small fraction (~20%) of neurons. Images were acquired with an EMCCD (electron-multiplying charge-coupled device) camera (frame time: 5–50 ms) and a stabilized LED light source of cultures visualized through a coverslip with a high-NA objective (Zeiss; 63×/1.4 NA; NA). All experiments were performed in TRIS buffered saline (1 ml/h) and high-affinity versions of SF-ΔGluSnFR were expressed in a similar manner.

Localization of glutamate-release sites. Primary hippocampal neurons were transduced with AAV2/1.hSynapsin1.SF-ΔGluSnFR.R5T2A or AAV2/1.hSynapsin1.SF-ΔGluSnFR.A184V, at 1013 GC/ml in vitro (DIV) using a DIV 9.
A low-amplitude electrical field stimulation (1 ms, 20 mA; platinum bar electrodes) was applied to activate a small fraction (~20%) of neurons only. Per experiment, stimulation was applied multiple times at an interstimulus interval of 60 s. Slices were acquired with an EM-CCD camera (Hamamatsu Image X1; 8×-magnification, 125–Hz acquisition rate) attached to an inverted microscope (Nikon Ti Eclipse) using a triggered, stabilized LED light source (Cairn OptoLED with 470-nm excitation wavelength, 470/60 emission filter and S25/50 excitation filter). Cells were imaged through a coverslip with a high-NA objective (Zeiss; 63×/1.4-NA, water). All experiments were performed in saline (1 ml/min, as described above) at room temperature.

In each experiment 30 images were acquired per stimulation trial (20 before and 10 after stimulation). Each of the 30 images was registered to the first image with the StackReg plugin in ImageJ. The image series was then normalized to the average of the first image to distinguish responding sites (~1) and nonresponding structures (~0). For the selection of responding sites to be included in the analysis, ten normalized images subsequent to the stimulus in the first trial were averaged. All spots of increased fluorescence (Supplementary Fig. 10c,d) that reached at least 50% of the ΔF/F value of the brightest spot in the image were defined as responding spots and used for further analysis. We quantified the spatial extent of glutamate-release sites by extracting a brightness profile based on a line (length, 12–30 pixels; width, 3 pixels) drawn along the underlining neurite. These profiles were calculated for each stimulation trial and each responding site in an experiment and fitted by Gaussians with Igor Pro 6.3 (WaveMetrics).

In each experiment n = 6 and 8 for S72A and A184V, respectively, each consisting of 16–25 trials the mean deviation of the center (X-position), the average width and the average amplitude of the fitted Gaussians were calculated per response site and averaged across all experiments and statistically compared by unpaired Mann–Whitney test (n = 28 and 53 for S72A and A184V, respectively).

Cerebellar granule cell axon experiments and analysis. All protocols were approved by the ethics committee CEEA-Paris1.

Stereotaxic injections. To fluorescently label boutons of parallel fibers, we administered stereotaxic injections of viral vectors expressing SF-GluSnFR or GaCaMP6f into cerebellar vermis. We used the following vectors: AAV-DJ-hSynapsin1-5F–GluSnFR (1.9 × 1013 GC/ml), AAV-DJ-hSynapsin1-5F–GluSnFR.S72A (2.6 × 1013 GC/ml), AAV-DJ-CAG.FLEX SF–GluSnFR.S72A (6.3 × 1013 GC/ml) and AAV-DJ-hSynapsin1-5F–GaCaMP6f (1.2 × 1013 GC/ml). Mice between 3 and 8 weeks of age were anesthetized with a mixture of hypnotic (ketamine 1.5%; Mérial) and analgesic (xylazine 0.05%; Labsynth) and placed in a stereotaxic frame adaptor comprising adjustable ear bars and tooth holder. The skull was then perforated at the injection site with a surgical drill. The vermis was identified by the cranial incision. The anesthetized mouse was then placed on a stereotaxic stage and fixed in a stereotaxic frame. After preparation, the slices were incubated at 32 °C for 30 min in the following mixture of hypertonic (ketamine 1.5%; Mérial) and analgesic (xylazine 0.05%; Labsynth) (equation (2)) of partial phototransient and its variance (equation (3))

where t0 is the time of the peak of fluorescence responses was calculated from fluorescence traces that were corrected only for photomultiplier tube bias current (F(t)) and using equation (3):

The trial-averaged fluorescence transient (⟨F(t)⟩) and its variance (σ_F(t)) were calculated from 50 to 70 trials (0.5 Hz) for each bouton. (ΔF) is the mean peak amplitude calculated from a 20 ms-window around the peak of (F(t)) corrected by the mean of a 20 ms-window baseline before the start of the electrical stimulation. σ_F was calculated as the mean variance of (σ_F(t)) during the peak window. The dark noise (σ_D) was measured without laser illumination. The final variance was estimated after subtraction of the expected increased variance due to photodetector shot noise (σ_D), which itself was estimated by scaling of the fit (equation (2)) of (F(t)) scaled to the average value calculated from the baseline window of (σ_F(t)).
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For electroporation, 1 µg of DNA was mixed with 1 × 10^10 cells using the Amaxa Nucleofector II instrument. Cells were plated onto coverslips coated with poly-D-lysine (Sigma) and kept at 37 °C, 5% CO2 in plating media for ~24 h, and then Nucleofector II instrument. Cells were plated onto coverslips coated with poly-D-lysine (Sigma) and kept at 37 °C, 5% CO2 in plating media for ~24 h, and then Nucleofector II instrument. Cells were plated onto coverslips coated with poly-D-lysine (Sigma) and kept at 37 °C, 5% CO2 in plating media for ~24 h, and then

Glutamate uncaging and imaging. Rat hippocampal culture was imaged on DIV 19 at room temperature in HEPES-buffered Tyrode’s solution (145 mM NaCl, 2.5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM CaCl2, 1 mM MgCl2, pH 7.4).

Excitation was applied with a 1,030-nm, 5-MHz, 190-fs laser (Menlo Systems; Bluecut). The average power was 39 mW at the sample. Fluorescence was collected at 560/80 nm with a Hamamatsu MPPC detector. The field of view was a 256-μm-diameter circle 1,280 pixels across. The bath contained HEPES-buffered Tyrode’s solution plus 10µM NBQX and 150µM RuBi-Glutamate (Tocris). Glutamate uncaging was performed with 420-nm fiber-coupled LEDs (Thorlabs; M420F2). The tips of the fibers were imaged onto the sample plane through the same objective used for activity imaging.

Imaging SF-Venus-iGluSnFR at 1,030 nm in vivo. All surgical procedures were in accordance with protocols approved by the HHMI Janelia Research Campus Institutional Animal Care and Use Committee (IACUC 17–155). An 8-week-old female C57BL/6NCrl mouse was anesthetized with isoflurane in oxygen and placed on a heated pad, and its head was gently affixed to a head holder by its front teeth. Buprenorphine HCl (0.1 mg/kg) and ketoprofen (5 mg/kg) were administered to the animal via subcutaneous injection. A 4.5-mm craniotomy (centered ~3.5 mm lateral, −0.5 mm rostrally from lambda) was opened. We administered 6–8 injections (30 nl each, 1 nl/s, 300 µm deep) of viral suspension containing AAV2/1:Synapsin1.FLEX.SF-Venus-iGluSnFR.A1845 (final titer: 5 × 10^10 GC/mL), AAV9:CaMKIIa.Cre (final titer: 5 × 10^10 gc/ml), final titer 1 × 10^10 gc/ml, to the left visual cortex. The craniotomy was closed with a 4-mm round coverslip that was centered at 65° of azimuth and −10° of elevation. A high-extinction 500-nm shortpass filter (Wright 47B-type) was affixed to the screen. Stimuli were drifting square-wave gratings (0.153 cycles per cm, 1 cycle s⁻¹, 2-s duration) in eight equally spaced directions, spaced by periods of mean luminance, as measured using the microscope detection path. Fluorescence was collected in two channels (500–580 nm and 595–685 nm) in a custom-built detection system. Isolated dendrites were imaged at 3.4 Hz. Images were aligned to compensate for bidirectional scanning and rolling-shutter motion artifacts using a series of strip-wise registrations implemented in MATLAB code, available upon request. A region of interest was selected manually corresponding to a single dendritic spine. The baseline F0 was calculated as the mean fluorescence intensity over 1 s before stimulus onset.

Simulations of glutamate diffusion. A finite differencing model was used to simulate diffusion of glutamate in water (diffusivity 700 μm²/s), with an isotropic spatial resolution of 200 nm, from an initial distribution determined from a 3D intensity pattern that approximated the uncaging light pattern used in our experiments (5-μm spot, 0.8%NA). A time step of 1.43µs was used, which ensured stability of the simulation with these parameters. The data were fit with custom MATLAB code (glutamateDiffusion.m), available upon request.

Statistical tests. Data are presented as mean and s.e. or s.d., as noted in each case. All n values are provided in the figure legends; no data were excluded. For comparisons between two datasets, either a two-sided Student’s t-test or two-sided Mann–Whitney U-test (a.k.a. Wilcoxon rank-sum test) was used. For comparisons of three or more datasets, Kruskal–Wallis and post hoc Dunn’s multiple-comparisons tests were used.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code availability. All analysis code used in this study is available upon request.

Data availability

All data from this study are available upon request. All constructs have been deposited at Addgene (#106174–106206; hSynapsin1, FLEX-hSynapsin1, FLEX, CAG, GFAP promoters; some fusions with the red fluorescent protein mRuby3 are available). Sequences have been deposited in GenBank (MH392460, MH392461, MH392462, MH392463, MH392464, and MH392465). AAV is available from Addgene.

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## Experimental design

1. **Sample size**
   - Describe how sample size was determined.
   - No statistical methods were used to determine sample size. Experiments were performed enough times to achieve consistent results and permit statistical analysis when appropriate.

2. **Data exclusions**
   - Describe any data exclusions.
   - No data points were excluded.

3. **Replication**
   - Describe whether the experimental findings were reliably reproduced.
   - No attempted replications failed. All experiments are shown.

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - Randomization is not relevant to this study because no comparisons between experimental groups or treatments were made.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - No blinding was performed because these experiments were performed to optimize and characterize a molecular toolset, not test a hypothesis.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**
   - For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   | n/a | Confirmed |
   |-----|-----------|
   | ☐   | ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
   | ☐   | ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
   | ☐   | ☒ A statement indicating how many times each experiment was replicated |
   | ☐   | ☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
   | ☐   | ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
   | ☐   | ☒ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted |
   | ☐   | ☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
   | ☐   | ☒ Clearly defined error bars |

*See the web collection on statistics for biologists for further resources and guidance.*
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

ScanImage, ImageJ, Spike2, PsychoPy, MATLAB, Igor Pro, custom code for the FADE algorithm (accepted preprint attached, code available).

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials are available.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

No antibodies were used.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

The only cell line used was HEK293 (ATCC), used for production of adeno-associated virus.

b. Describe the method of cell line authentication used.

HEK293 is periodically acquired directly from ATCC.

c. Report whether the cell lines were tested for mycoplasma contamination.

Cell lines were routinely checked to ensure authenticity and absence of mycoplasma infection.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Neuronal cultured were prepared from Sprague-Dawley rat pups (Charles River Laboratories; P0 pups, either sex), or from E16 C57BL/6J mice (Charles River, either sex). For cerebellar parallel fiber imaging, adult, either-sex CB6F1 mice (F1 cross of BalbC and C57BL/6J) or adult, either-sex Gabra6 mice (B6;129P2-186 Gabra6tm2(cre)Wwis/Mmucd) were used (viral injection postnatal day 30 and 60, slices cut postnatal day 41 to 123). For mouse in vivo imaging, C57/B6J mice (Jackson Labs, either sex, AAV-injected postnatal 8 weeks) were used. For ferrets, juvenile female ferrets aged P21-P22 (n=2) (Mustela putorius furo, Marshall Farms) were used.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not involve human participants.