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Supplemental Information

BATTLE: Genetically Engineered Strategies for Split-Tunable Allocation of Multiple Transgenes in the Nervous System

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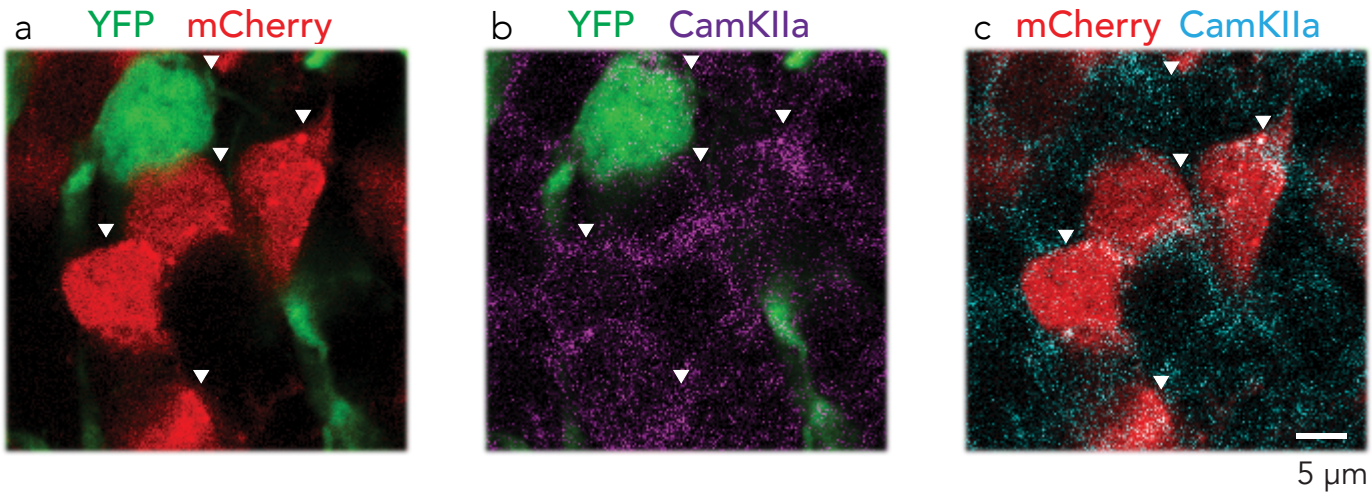

Figure S1. Specific transgene expressions of YFP and mCherry in excitatory neurons, Related to Figure 1.

a, Representative confocal image of dentate gyrus in hippocampus injected with BATTLE-1. Arrowheads indicate YFP-positive and mCherry-positive granule neurons. **b**, Specific transgene expression of YFP in CamKIIa expressing excitatory granule neurons. Magenta color shows CamKIIa immunoreactivity. **c**, Specific expression of mCherry in CamKIIa expressing excitatory granule neurons. Cyan color shows CamKIIa immunoreactivity. Scale bar represents 5 μm.

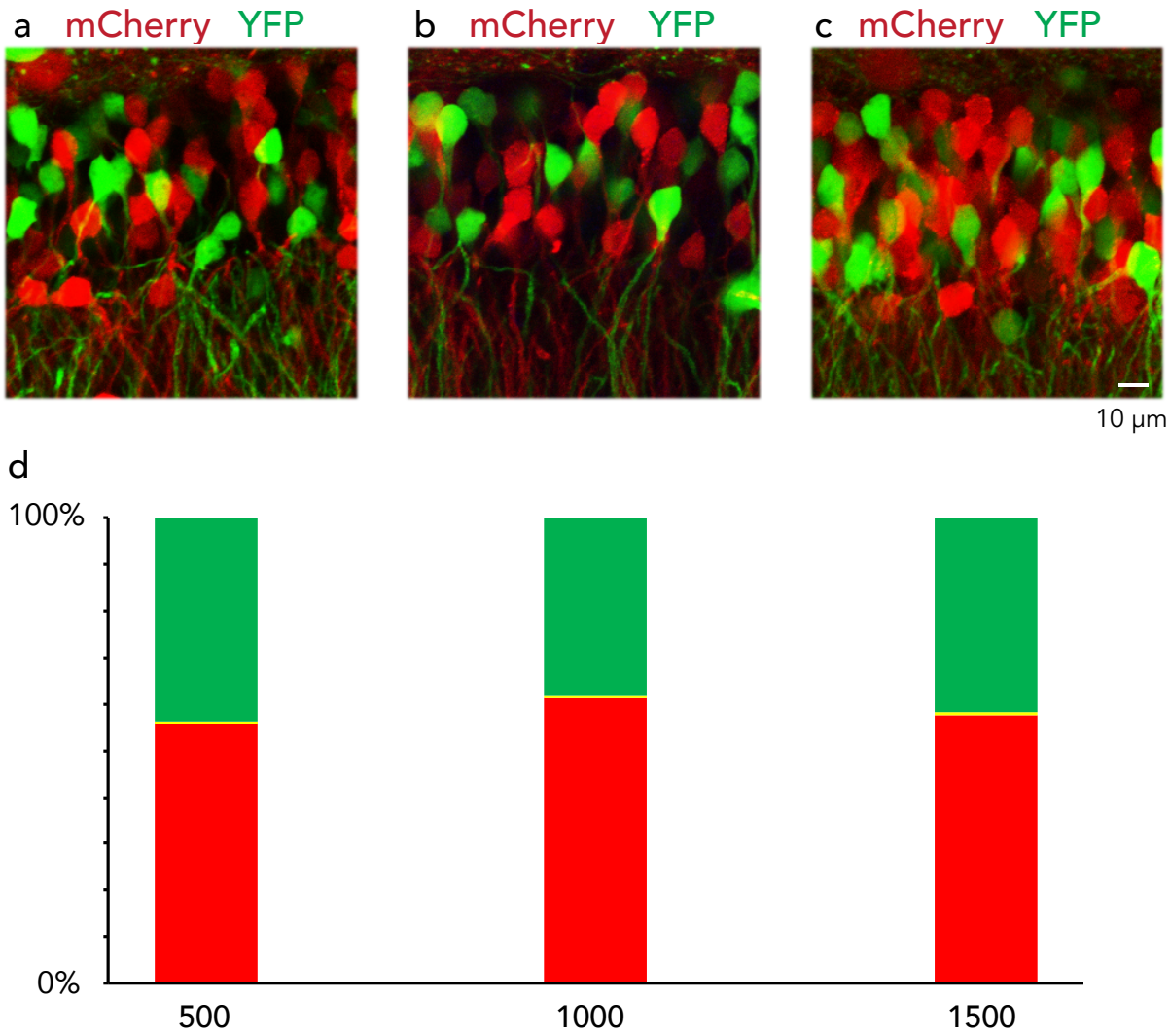

Figure S2. Splitting allocations of YFP and mCherry to granule neurons injected with BATTLE-1 at 500, 1000, and 1500 nL dosages, Related to Figure 1.

a-c, Representative maximum intensity projection image of dentate gyrus in the hippocampus injected with BATTLE-1 at 500, 1000, and 1500 nL dosages of mixed lentiviruses. Scale bar represents 10 μm. **d**, Quantification of the percentage of cells expressing fluorescent proteins in the dentate gyrus injected with BATTLE-1 (left, 500 nL; middle, 100 nL; right, 1500 nL). Red, green, and yellow represent the percentage of cells expressing mCherry-positive, YFP-positive, and YFP- and mCherry- double-positive granule neurons, respectively. For the 1000 nL dosage, the percentage of cells that were mCherry-positive, YFP-positive, and YFP- and mCherry- double positive were 61.3, 38.0, and 0.7%, respectively.

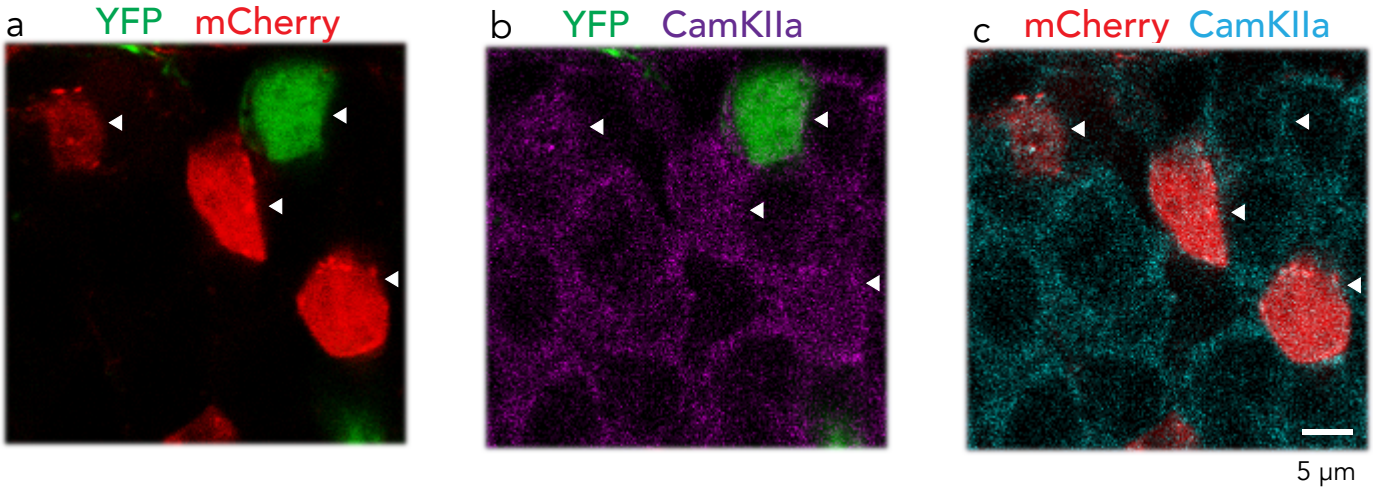

Figure S3. Specific transgene expressions of YFP and mCherry in excitatory neurons injected with *BATTLE*-2, Related to Figure 2.

a, Representative confocal image of dentate gyrus in the hippocampus injected with *BATTLE*-2. Arrowheads indicate YFP-positive and mCherry-positive granule neurons. **b**, Specific transgene expression of YFP in CamKIIa expressing excitatory granule neurons. Magenta represents CamKIIa immunoreactivity. **c**, Specific expression of mCherry in CamKIIa expressing excitatory granule neurons. Cyan represents CamKIIa immunoreactivity. Scale bar represents 5 μm.

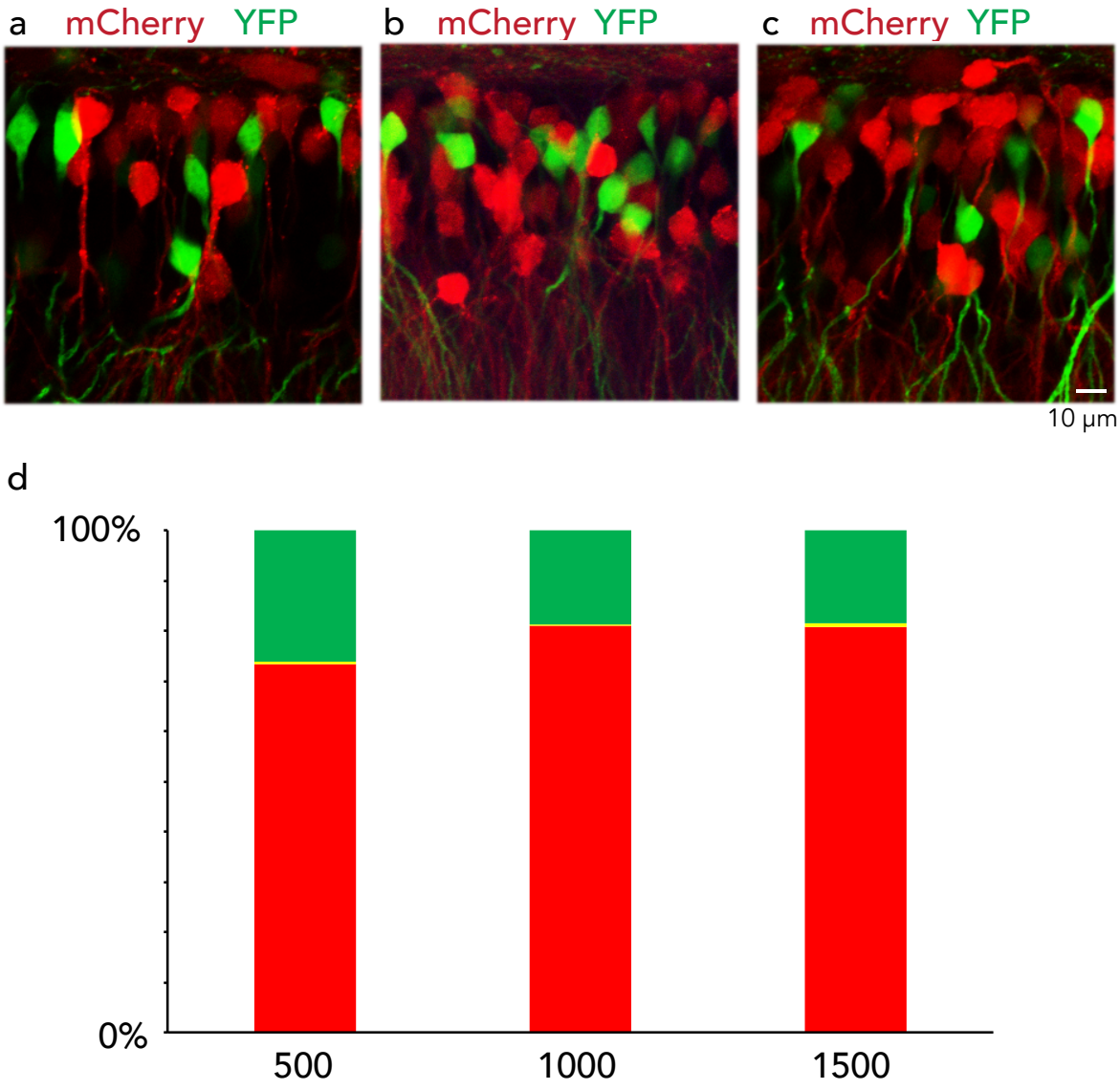

Figure S4. Splitting allocations of YFP and mCherry to granule neurons injected with *BATTLE*-2 at 500, 1000, and 1500 nL dosages, Related to Figure 2.

a-c, Representative maximum intensity projection images of dentate gyrus in the hippocampus injected with *BATTLE*-2 at 500, 1000, and 1500 nL dosages of mixed lentiviruses. Scale bar represents 10 μm. **d**, Quantification of the percentage of cells expressing fluorescent proteins in the dentate gyrus injected with *BATTLE*-2 (left, 500 nL; middle, 1000 nL; right, 1500 nL). Red, green, and yellow represent the percentage of cells expressing mCherry-positive, YFP-positive, and YFP- and mCherry- double-positive granule neurons, respectively.

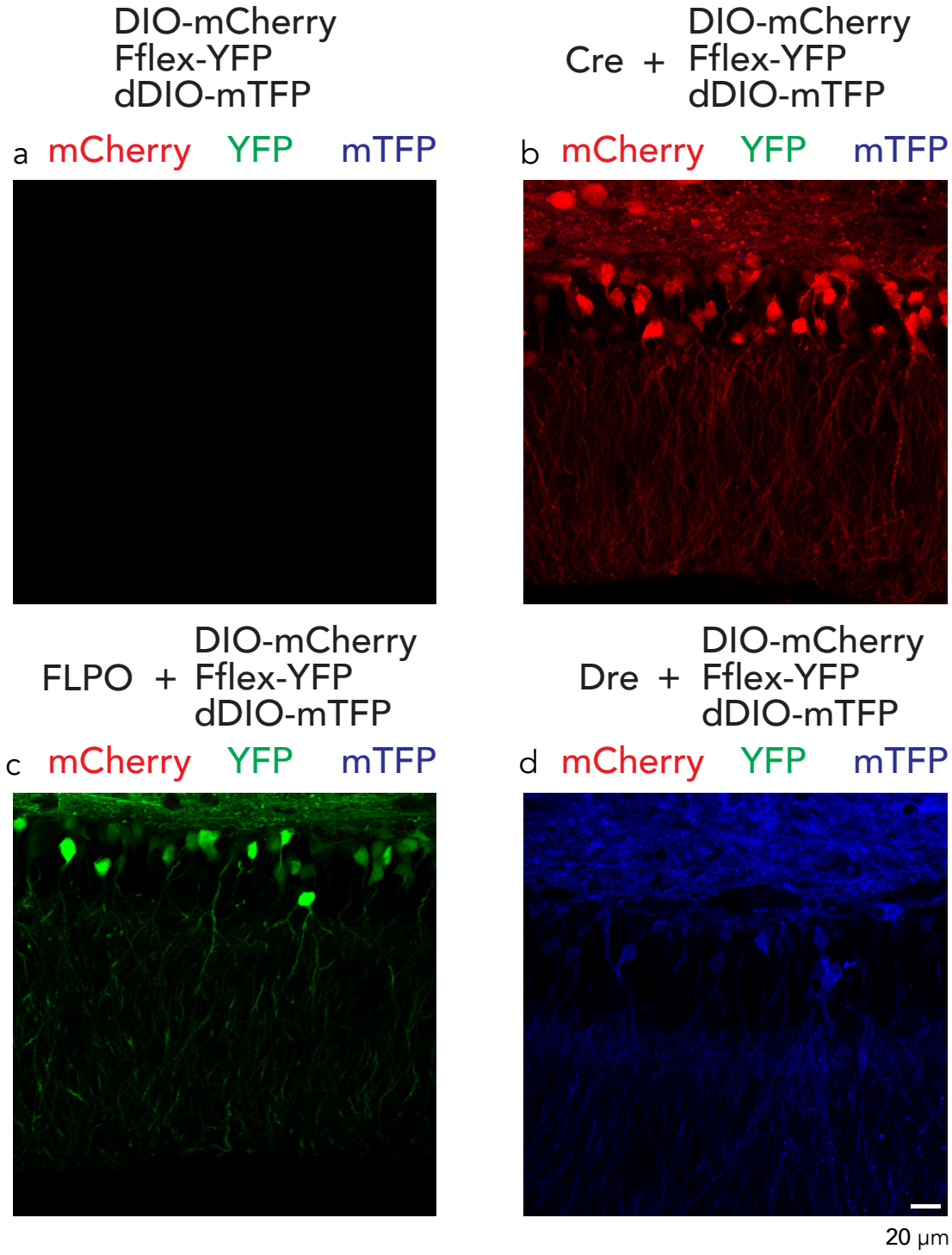

Figure S5. Recombinase-specific transgene expression of Cre-, FLPO-, and Dre-dependent AAV, Related to Figure 3.

a, Representative confocal image of dentate gyrus in hippocampus after injections of AAV-Ef1a-DIO-mCherry, AAV-EF1a-F-flex-YFP, and AAV-Ef1a-dDIO-mTFP. **b**, Representative confocal image of dentate gyrus in hippocampus after injections of lentivirus expressing Cre recombinase, AAV-Ef1a-DIO-mCherry, AAV-EF1a-F-flex-YFP, and AAV-Ef1a-dDIO-mTFP. **c**, Representative confocal image of dentate gyrus in hippocampus after injections of lentivirus expressing FLPO recombinase, AAV-Ef1a-DIO-mCherry, AAV-EF1a-F-flex-YFP, and AAV-Ef1a-dDIO-mTFP. **d**, Representative confocal image of dentate gyrus in hippocampus after injections of lentivirus expressing Dre recombinase, AAV-Ef1a-DIO-mCherry, AAV-EF1a-F-flex-YFP, and AAV-Ef1a-dDIO-mTFP. Scale bar represents 20 µm.

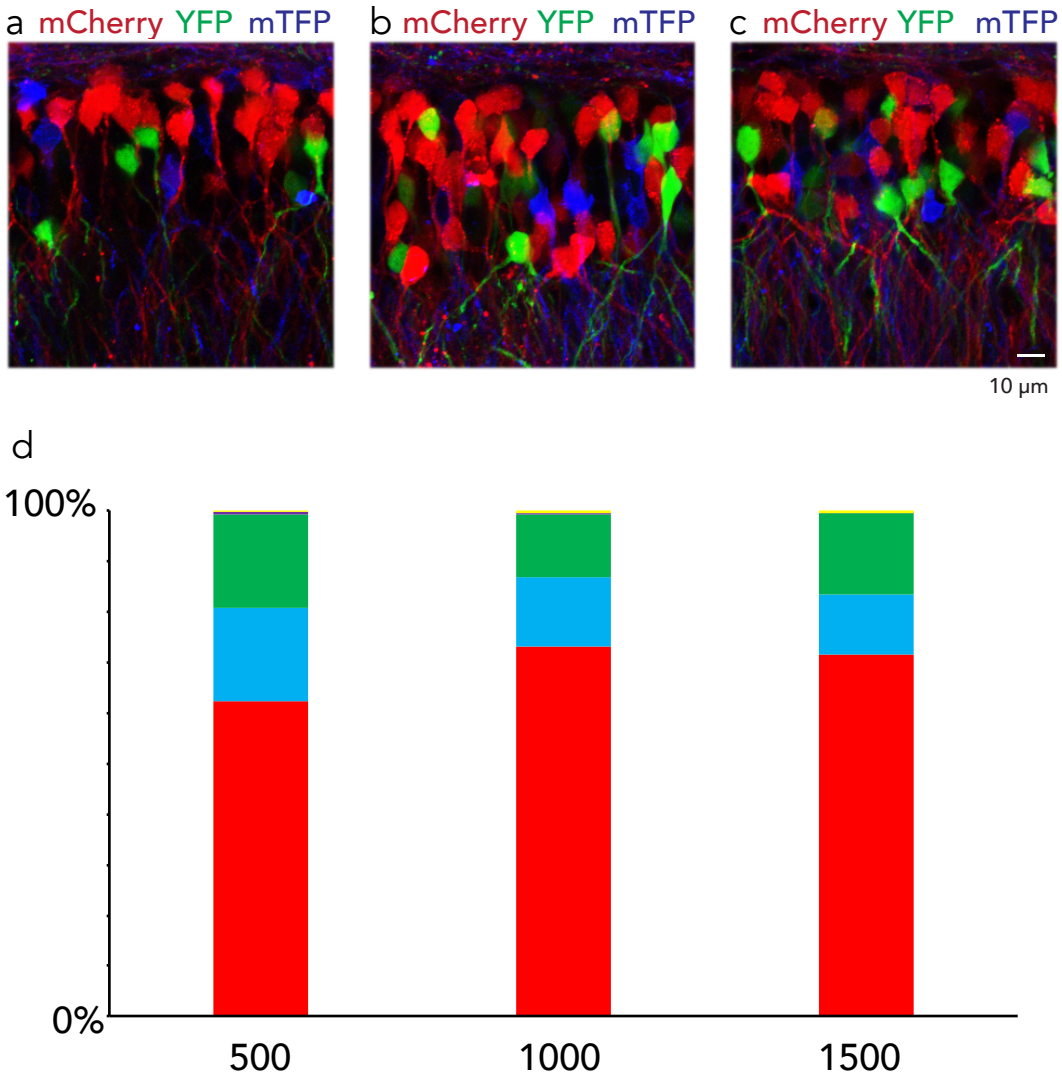

Figure S6. Triple splittable allocations of YFP, mTFP, and mCherry to granule neurons injected with *BATTLE*-2.1 at 500, 1000, and 1500 nL dosages, Related to Figure 3.
a-c, Representative maximum intensity projection images of dentate gyrus in the hippocampus injected with *BATTLE*-2.1 at 500, 1000, and 1500 nL dosages of mixed lentiviruses. Scale bar represents 10 μm. **d**, Quantification of the percentage of cells expressing fluorescent proteins in the dentate gyrus injected with *BATTLE*-2.1 (left, 500 nL; middle, 100 nL; right, 1500 nL). Red, blue, green, yellow, and purple represent the percentage of cells expressing mCherry-positive, mTFP-positive, YFP-positive, YFP- and mCherry- double-positive, and mTFP- and mCherry- double-positive granule neurons, respectively.

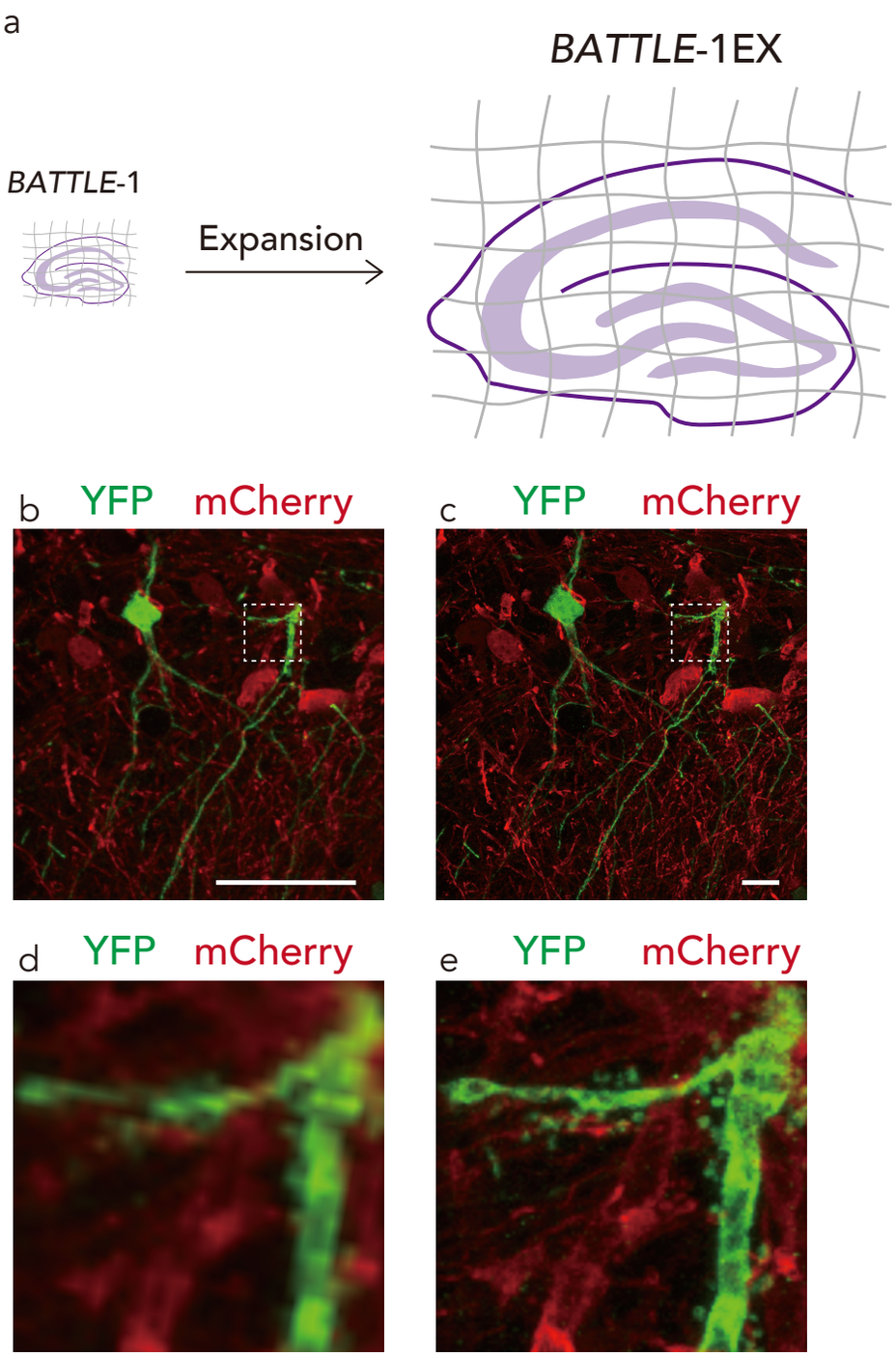

Figure S7. Comparison of sample images between pre- and post-expansion in *BATTLE*-1EX, Related to Figure 4.

a, Schematic images of pre- and post-expansion samples in *BATTLE*-1EX. **b and c**, Representative maximum intensity projection 2D images of hippocampal pyramidal neurons were compared between pre-expansion (b) and post-expansion (c). Scale bars: 50 μm. **d and e**, Magnified views of boxed regions in (b) and (c), respectively.

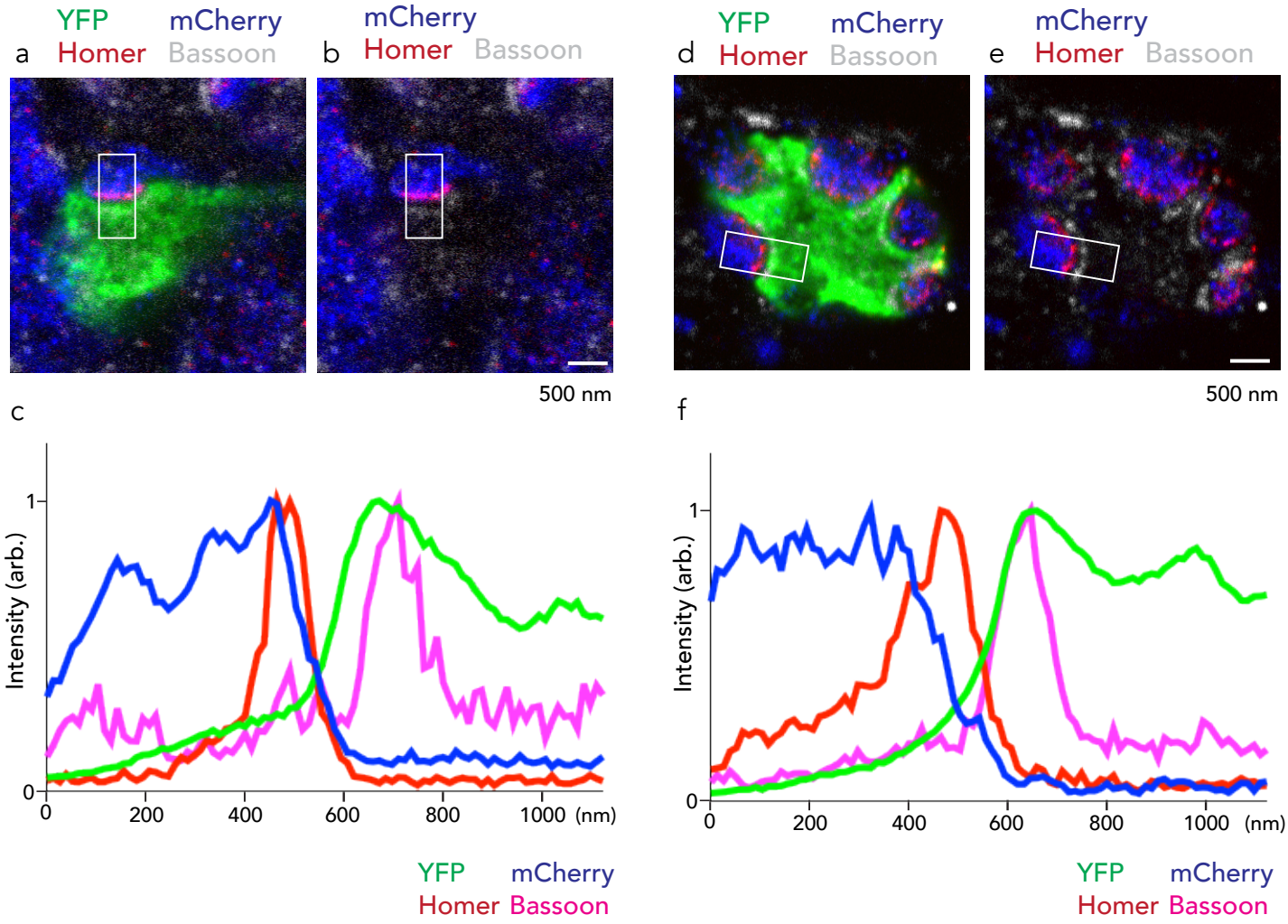

Figure S8. Other representative images of high-resolution imaging of DG-CA3 synapses in the hippocampus using *BATTLE*-1EX, Related to Figure 4.

a and **b**, Representative confocal image of DG-CA3 synapses using *BATTLE*-1EX. **c**, Line profiling analysis of boxes in a and b. **d** and **e**, Another representative confocal image of DG-CA3 synapses using *BATTLE*-1EX. **f**, Another line profiling analysis of boxes in d and e. Scale bars represent 500 nm.

Transparent Methods

Experiments were conducted in accordance with Kansai Medical University regulations and approved by the committee on Animal Care. Mice were housed as 2–5 littermates per cage with a regular dark-light cycle and access to water and food. Post-surgery mice were individually housed.

Generation of viruses

To create the lentivirus-CamKIIa (1.3 kb)-FRT5-iCre-FRT5-WPRE vector, a synthesized transgene FRT5-iCre-FRT5 was inserted into the BamHI-EcoRI site of the lentiviral backbone vector (Addgene 20943). Then, a synthesized transgene loxN-FLPO-loxN was also inserted into the BamHI-EcoRI site of the lentiviral backbone vector (Addgene 20943) to create lentivirus-CamKIIa(1.3 kb)-loxN-FLPO-loxN-WPRE vector. For *BATTLE*-2 viral Cre vectors, a synthesized transgene BamHI-FRT5-rox-AgeI was inserted into the BamHI-AgeI site of lentivirus-CamKIIa(1.3 kb)-FRT5-iCre-FRT5-WPRE vector.

Then, a synthesized transgene NheI-rox-FRT5-EcoRI was inserted into the Nhe-EcoRI site of lentivirus-CamKIIa(1.3 kb)-FRT5-rox-iCre-FRT5-WPRE vector. For *BATTLE*-2 viral FLPO vectors, synthesized transgene BamHI-loxN-rox-AgeI was

inserted into the BamHI-AgeI site of lentivirus-CamKIIa (1.3 kb)-loxN-FLPO-loxN-WPRE vector. A synthesized transgene NheI-rox-loxN-EcoRI was inserted into the NheI-EcoRI site of lentivirus-CamKIIa (1.3 kb)-loxN-rox-FLPO-loxN-WPRE vector to create the lentivirus-CamKIIa(1.3 kb)-loxN-rox-FLPO-rox-loxN-WPRE vector.

For *BATTLE*-2 viral shadow Dre vectors, a synthesized transgene BamHI-FRT5-loxN-Dre-LoxN-FRT5-EcoRI was inserted into the BamHI-EcoRI site of the lentivirus-CamKIIa (1.3 kb) backbone vector to create the lentivirus-CamKIIa (1.3 kb)-FRT5-loxN-Dre-LoxN-FRT5-WPRE vector. These five lentiviruses were packaged at Gunma University. For AAV vectors, a synthesized transgene NheI-YFP-BSrGI was inserted into the NheI-BSrGI site of AAV F-Flex backbone vector (Addgene 60661) to create the AAV-EF1a-F-flex-YFP vector. A synthesized transgene KpNI-Rox12-Rox2-speI-reverse complement mTFP-reverse complement Rox12-reverse complement Rox2-EcoRI was inserted into the KpnI-EcoRI site of pAAV-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA vector (Addgene 20298) to create AAV-Ef1a-dDIO-mTFP. AAV-Ef1a-DIO-mCherry (Addgene Plasmid 47636), AAV-EF1a-F-flex-YFP, and AAV-Ef1a-dDIO-mTFP were packaged at Gunma University.

Stereotaxic injections of viruses

Mice were anesthetized with isoflurane and placed in a stereotaxic apparatus (World Precision Instruments). Tapered glass capillaries were processed by Micropipette Puller (Sutter). The tapered glass capillary and Hamilton syringe with a 26-gauge needle were filled with mineral oil and used to inject the viruses that were injected into the hippocampal DG-CA2-3 area in the right hemisphere at the predetermined coordinates (-1.94 mm AP, +2.14 mm ML, -2.32 mm DV) from the bregma. For *BATTLE*-1 viruses, adult mice were infected with 1 μL mixed lentiviruses (lenti-CamKIIa-FRT5-Cre-FRT5:lenti-CamKIIa-loxN-FLPO-loxN=1:1 [each virus consisted of 5×10E8 copies] or lenti-Cre:lenti-FLPO=1:0.01 [5×10E8 and 5×10E6 copies, respectively]) with the first injections. For *BATTLE*-1 virus dosage experiments, adult mice were infected with 500 nL or 1500 nL mixed lentiviruses (lenti-CamKIIa-FRT5-Cre-FRT5:lenti-CamKIIa-loxN-FLPO-loxN=1:1).

Three weeks later, AAV-EF1a-DIO-mCherry (1 μL, titer: 1.43×10E13) and AAV-EF1a-DIO-YFP (1 μL, titer: 1.43×10E13) were mixed and injected at the same sites used for the first injections. The mice were then perfused 1 week after the second injections. For *BATTLE*-2 virus injections for splitting transgene expression, adult mice were infected with *BATTLE*-2 lentiviruses (1 μL, Lenti-CamKIIa-FRT5-rox-Cre-FRT5:lenti-CamKIIa-loxN-rox-FLPO-rox-loxN:lenti-CamKIIa FRT5-loxN-Dre-loxN-

FRT5=1:1:1, each virus consisted of 3×10E8 copies) with the first injections. For *BATTLE*-2 virus dosage experiments, adult mice were infected with 500 nL or 1500 nL mixed lentiviruses (Lenti-CamKIIa-FRT5-rox-Cre-FRT5:lenti-CamKIIa-loxN-rox-FLPO-rox-loxN:lenti-CamKIIa-FRT5-loxN-Dre-loxN-FRT5=1:1:1). Three weeks later, AAV-EF1a-DIO-mCherry and AAV-EF1a-DIO-YFP (both 1 μL, titer: 1.43×10E13) were mixed and injected at the same sites used for the first injections. The mice were perfused 1 week after the second injections. For *BATTLE-2.1* triple-color virus experiments, adult mice were infected with 500 nL or 1000 nL or 1500 nL mixed lentiviruses (Lenti-CamKIIa-FRT5-rox-Cre-FRT5:lenti-CamKIIa-loxN-rox-FLPO-rox-loxN:lenti-CamKIIa FRT5-loxN-Dre-loxN-FRT5=1:1:1). Three weeks later, AAV-EF1a-DIO-mCherry, AAV-EF1a-fFlex-YFP, and AAV-Ef1a-dDIO-mTFP (all 667 nL, titer: 1.81×10E13) were mixed and injected at the same sites used for the first injections. For crosstalk control experiments, AAV-EF1a-DIO-mCherry, AAV-EF1a-fFlex-YFP, and AAV-Ef1a-dDIO-mTFP (all 667 nL, titer: 1.81×10E13) were mixed and injected into the hippocampus. The mice were then perfused 1 week after the second injections.

For crosstalk recombinase experiments, adult mice were infected with 1 μL lenti-CamKIIa-FRT5-loxN-Dre-loxN-FRT5 or lenti-CamKIIa-FRT5-rox-Cre-rox-FRT5 or lenti-CamKIIa-loxN-rox-FLPO-rox-loxN (viruses consisted of 0.5×10E9 copies). Then,

AAV-EF1a-DIO-mCherry, AAV-EF1a-fFlex-YFP, and AAV-Ef1a-dDIO-mTFP (all 667 nL, titer: 1.81×10E13) were injected on the same day as the first injections. The mice were then perfused 1 week after the second injections.

For *BATTLE*-2 virus injections of multi-sparse transgene expressions, adult mice were infected with 1 μL lenti-CamKIIa-FRT5-loxN-Dre-loxN-FRT5:lenti-CamKIIa-FRT5-rox-Cre-FRT5:lenti-CamKIIa-loxN-rox-FLPO-rox-loxN=200:1:10 (viruses consisted of 1×10E9, 5×10E6, and 5×10E7 copies, respectively). Then, AAV-EF1a-DIO-mCherry and AAV-EF1a-DIO-YFP (1 μL, titer: 1.43×10E13) were injected on the same day as the first injections. The mice were perfused 2 weeks after the viral injections. Adult mice were infected with conventional AAV-CAG-GFP viruses (Addgene 37825-AAVrg, 1 μL, titer 1×10E13) and then they were perfused 7 days later.

Immunohistochemistry

Adult mice were anesthetized with a mixture of medetomidine, midazolam, and butorphanol, and then transcardially perfused with ice-cold 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) using a peristaltic pump. Then, the brains were post-fixed in the same solution for 24 h, transferred to 30% (w/) sucrose in PBS, and embedded in optimal cutting temperature compound (Sakura). The brains were then

frozen with isopentane and liquid nitrogen and sectioned using a cryostat (Leica, 60 μm-thick sagittal sections).

For CamKIIa staining, freely floating sections were incubated with 3% (v/v) goat serum in 0.3% Triton X-PBS for 1 h, followed by incubation with primary mouse monoclonal antibody against CamKIIa (1:300, Abcam ab22609) in 3% goat serum plus 0.3% Triton X-PBS overnight at 4 °C. After rinsing the sections three times with 0.3% Triton X-PBS for 15 min, they were incubated with secondary anti-mouse goat antibody conjugated with Alexa Fluor 647 Plus (1:1000, A32728, Thermo Fisher Scientific) for 2 h.

Then, the sections were rinsed three times with 0.3% Triton X-PBS for 15 min. Co-expression of CamKIIa and fluorescent proteins was determined based on their expressions in the cytoplasm of cell bodies in confocal images captured using an Olympus confocal microscope (FV3000). All sections were mounted on glass slides using Vector shield and visualized using 4',6-diamidino-2-phenylindole (DAPI, Vector Lab).

Fluorescence imaging

Fluorescence images were acquired using an inverted confocal fluorescent microscope (Zeiss, LSM700, LSM710 and Olympus FV3000) using 10×, 20×, 40×, 60×,

and 63× objectives. Maximum intensity projection images were generated using Zenblack software (Zeiss), FV31S-SW software (Olympus), and Imaris 9.1 (Bitplane). 3D volume rendering (Figs. 3e, f, and 4) was generated after background subtraction using Imaris 9.1 (Bitplane).

Analysis of splitting expression of fluorescent protein

To analyze the splitting allocation of YFP and mCherry, fluorescent images were captured using confocal microscopy (Zeiss LSM700) with a 20× objective lens. To analyze the splitting allocation of mTFP, YFP, and mCherry in triple-color *BATTLE*-2.1 experiments or crosstalk experiments, fluorescent images were captured using confocal microscopy (Zeiss LSM710) with a 20× objective lens, 458 nm laser, 514 nm laser, and 561 nm laser. Fluorescence intensity was measured from square regions (3.9 μm × 3.9 μm) on the cell bodies of DG granule and CA2-3 neurons in the confocal images (400 μm × 400 μm × 2 μm) using the ZenBlue 2012 software (Zeiss). The co-expressions of mTFP, YFP, and mCherry were determined when both fluorescence intensities were higher than the background fluorescence (Arbitrary unit 10).

BATTLE-*1EX (expansion microscopy of* BATTLE-*1)*

ExM (expansion microscopy) was performed according a published method with minor modifications (Asano et al., 2018). Free-floating sections were subjected to antigen retrieval in citrate buffer (10 mM citric acid plus 0.05% Tween 20, pH 8.0) at 80 °C for 30 min, and washed four times with PBS for 5 min. Sections were permeabilized and blocked with blocking buffer containing 1% bovine serum albumin (BSA), 0.1% Triton X-100 in PBS for 1 h at 26 °C. Sections were then incubated with primary chicken polyclonal antibody to GFP (1:500, ab13970, Abcam), rat monoclonal antibody to RFP (1:100, 5f8-100, Chromotek), mouse monoclonal antibody to Bassoon (1:100, ab82958, Abcam), and rabbit polyclonal antibody to Homer1 (1:100, 160003, SYSY) in blocking buffer for 2 days at 4 °C, followed by washing four times with blocking buffer for 5 min.

Sections were incubated with secondary anti-chicken donkey antibody conjugated with Alexa488 dye (1:200, 703-545-155, Jackson), anti-rat donkey antibody conjugated with Alexa568 dye (1:100, ab175710, Abcam), anti-mouse goat antibody conjugated with Atto647N dye (1:100, 50185, Rockland), and anti-rabbit goat antibody conjugated with CF405M dye 1:100, 20373, Biotium) in blocking buffer for 1 day at 4 °C, washed sequentially three times each with blocking buffer, high-salt PBS (PBS+350 mM NaCl), and PBS for 5 min each time. Anchoring treatment was performed in Acryloyl-X

(0.1 mg/mL) in PBS for more than 6 h at room temperature, washed five times with PBS for 5 min each time.

For gelation, sections were immersed in the gelling solution (monomer solution [8.6% sodium acrylate, 2.5% acrylamide, 0.15% N,N′-methylenebisacrylamide, and 11.7% NaCl in PBS] mixed with ammonium persulfate, tetramethylethylenediamine, and 4-hydroxy-TEMPO at a ratio of 47:1:1:1) for 30 min on ice. Sections were then transferred to the gelling chamber (samples were sandwiched by a glass slide and cover slip on either side of the samples for spacers), and gelled for 2 h at 37 °C.

The hippocampal regions were trimmed from gelled sections and incubated with digestion buffer (50 mM Tris-HCl [pH 8.0], 23 mM ethylenediaminetetraacetic acid [EDTA], 0.5% Triton X-100, 0.8 M guanidine HCl, and 8 U/mL ProK) overnight, washed three times with PBS for 5 min, and then stored at 4 °C in the dark until expansion. For expansion, samples were washed five times with water for 10 min before transferring to the imaging chamber where they were sandwiched between a coverslip and silicon sheets, with silicon sheets on either side as spacers. The magnification was calculated using physical distances of two landmark positions in the pre- and post-expansion images measured by Imaris 9.1 (Bitplane).

Line profiling analysis of fluorescent intensities in whole synapses

To quantify whole synaptic structures and the distributions of Homer and Bassoon, measurements were conducted on a boxed area (478 nm × 1094 nm) of the whole synaptic area. The line profiling analysis of fluorescent intensities of YFP, mCherry, Homer, and Bassoon was performed using the Image J program (National Institutes for Health [NIH]).

Reproducibility

All experiments were conducted more than three times.