Title

Induction of input-specific spine shrinkage on dendrites of rodent hippocampal CA1 neurons using two-photon glutamate uncaging

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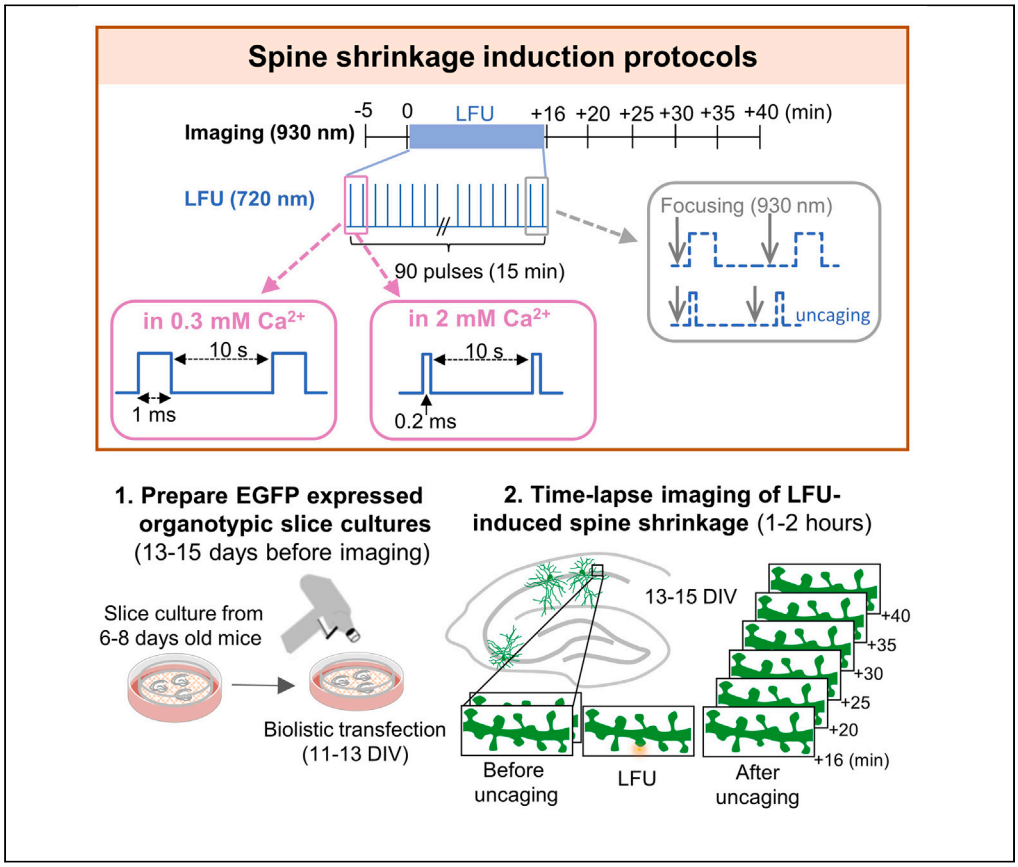

Shrinkage and loss of dendritic spines are vital components of the neuronal plasticity that supports learning. To investigate the mechanisms of spine shrinkage and loss, Oh and colleagues established a two-photon glutamate uncaging protocol that reliably induces input-specific spine shrinkage on dendrites of rodent hippocampal CA1 pyramidal neurons. Here, we provide a detailed description of that protocol and also an optimized version that can be used to induce input- and synapse-specific shrinkage of dendritic spines at physiological Ca^{2+} levels.

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Highlights

Two-photon photolysis of caged glutamate to induce dendritic spine shrinkage

Live imaging of sparsely labeled neurons using time-lapse two-photon microscopy

Quantitative analysis of dendritic spine size using fluorescence measurements

Preparation and transfection of organotypic hippocampal slice cultures

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Induction of input-specific spine shrinkage on dendrites of rodent hippocampal CA1 neurons using two-photon glutamate uncaging

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SUMMARY

Shrinkage and loss of dendritic spines are vital components of the neuronal plasticity that supports learning. To investigate the mechanisms of spine shrinkage and loss, Oh and colleagues established a two-photon glutamate uncaging protocol that reliably induces input-specific spine shrinkage on dendrites of rodent hippocampal CA1 pyramidal neurons. Here, we provide a detailed description of that protocol and also an optimized version that can be used to induce input- and synapse-specific shrinkage of dendritic spines at physiological Ca^{2+} levels. For complete details on the use and execution of this protocol, please refer to Oh et al. (2013), Stein et al. (2015), Stein et al. (2020), and Stein et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the steps for using two-photon glutamate uncaging to induce input-specific spine shrinkage on dendrites of CA1 neurons in organotypic hippocampal slice cultures from C57BL/6 mice of both sexes. However, we have also successfully implemented input-specific spine shrinkage using these protocols in Sprague-Dawley rats (Oh et al., 2013) and in acute slices from mice.

Preparation of media and tools for slice culture

Timing: 3 h

1. Prepare dissection medium and slice culture medium (SCM) (see materials and equipment). The dissection medium and SCM can be stored at 4°C for up to 2 weeks.
2. Autoclave dissection tools.

Preparation of organotypic cultured hippocampal slices

Timing: 90 min, 13–15 days before imaging

All experimental protocols were approved by the University of California Davis Institutional Animal Care and Use Committee.

Prepare organotypic hippocampal slices from postnatal day 6–8 C57BL/6 mice. Further details can be found in Opitz-Araya and Barria (2011).

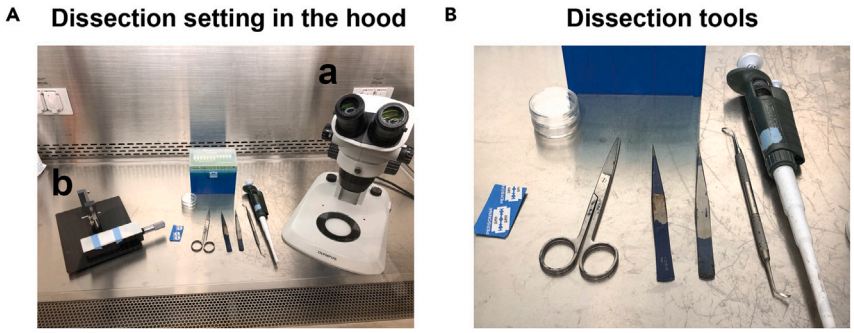

Figure 1. Tools for preparation of cultured hippocampal slices

(A) Place dissecting microscope (a) and tissue slicer (b) in the biosafety cabinet or sterile area for dissection. Disinfect by wiping with 70% EtOH and expose to UV light for ~ 15 min prior to dissection.

(B) Place autoclaved dissection tools (one surgical scissors, two sharp point forceps, and one dental scoop), two 35 mm plastic petri dishes, and one 1 mL pipette in the biosafety cabinet or sterile area for dissection.

3. In the biosafety cabinet, place tissue culture inserts into the wells of a six-well tissue culture plate with 900–1000 μL SCM per well and put in the tissue culture incubator with 5% CO_2 at 35°C for at least 30 min.
4. Place the dissecting microscope and tissue slicer, each disinfected with 70% ethanol, and the autoclaved dissecting tools into the hood (Figure 1). Turn on the UV lamp for 15 min.
5. Isolate the whole brain at the lab bench and place it into an ice-cold dissection medium aerated with 95% O_2/ 5% CO_2.
6. Turn off the UV lamp. In the biosafety cabinet, remove the hippocampi under the dissecting microscope and place them in a 35 mm petri dish filled with ice-cold dissection medium.

To remove the hippocampi, a sharp point forceps is used to hold the cerebellum and a dental scoop is used to scoop out the hippocampus.

7. Place isolated hippocampi on a Teflon sheet taped on the stage of a manual tissue slicer (Figure 1). To transfer the isolated hippocampi, use a 1000 μL pipetman with a plastic tip that is cut off at the end to increase the diameter. Remove solutions around isolated hippocampi carefully.
8. Cut 300 or 400 μm hippocampal slices perpendicular to the long axis of the hippocampus.
9. Return sliced hippocampi to a petri dish filled with cold SCM (4°C–8°C) and carefully separate individual slices.
10. Using a transfer pipette with a wide opening to avoid tissue damage, place 3–4 slices on each tissue culture insert in the six-well culture plate prepared in step 3. Remove excess liquid from around the slices on the inserts.
11. Place the culture plate in a tissue culture incubator with 5% CO_2 at 35°C.
12. Change SCM every 3–4 days.

Neuronal transfection for *in vitro* imaging preparations

Timing: 2 h, 2–3 days before imaging

Transfect cultured slices 2–3 days before imaging [11–15 Days In Vitro (DIV)] using biolistic transfection with plasmid encoding fluorescent protein. We typically use 10–15 μg EGFP coated onto 8 mg of 1.6 μm gold bullets, as described (Woods and Zito, 2008).

Alignment of uncaging and imaging lasers and laser power calibration

Timing: 30 min, immediately prior to imaging

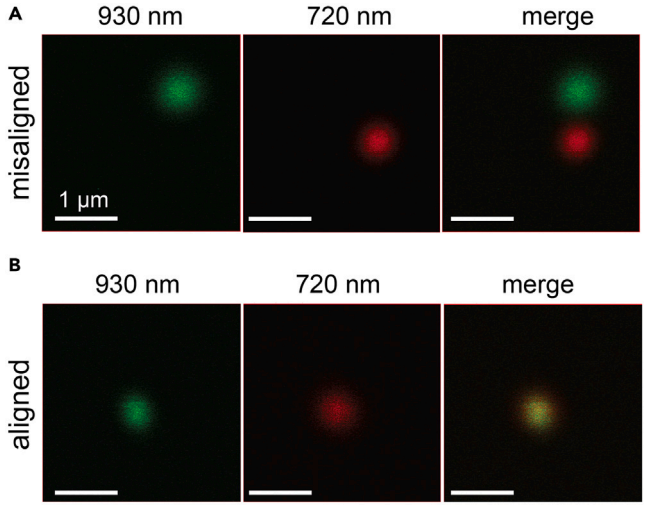

Figure 2. Laser alignment

(A) Image of a fluorescent bead when 930 and 720 nm lasers are misaligned. Left: Pseudocolored (green) image by 930 nm excitation. Middle: Pseudocolored image (red) by 720 nm excitation. Right: Merged images.

(B) Image of a fluorescent bead after laser alignment. Yellow color in the merged image (Right) shows overlap of 'green' and 'red' signals by 930 and 720 nm lasers, respectively.

13. Prepare microscope slides coated with sub-resolution (100–200 nm diameter) fluorescent beads (e.g., FluoSpheres F8801 or F8810, ThermoFisher).
    a. Dilute the beads 1:10,000 in water.
    b. Cover the surface of the slide with the diluted bead solution.
    c. Sprinkle NaCl powder into the diluted bead solution to precipitate the beads.
    d. Rinse off the solution and salt with water and let dry.
    e. Check density of beads using water immersion imaging objective. If beads are too high density to find isolated beads, increase bead dilution and make another slide.
14. With the 720 nm laser blocked or attenuated, focus on a well-separated individual bead at high zoom with the 930 nm laser. Minimize imaging time to prevent photobleaching.
15. Unblock the 720 nm laser. Align images of the bead from the 720 and 930 nm lasers until overlapping (Figure 2).
16. Measure laser power of 720 and 930 nm wavelengths at the sample plane using a handheld power meter (e.g., LaserCheck, Coherent) below the objective at several software settings (e.g., 0, 25, 50, 75, and 100% power). Plot measured laser powers (mW) against software power setting (%) to check the stability of the laser system.

Note: Alignment of lasers and laser power calibration should be performed every time after turning on the imaging system.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
FluoSpheres fluorescent beads	Thermo Fisher Scientific	F8801 or F8810
MEM Eagle medium	Sigma-Aldrich	M4642
Phenol red solution (0.5%)	Sigma-Aldrich	P20290
Horse serum, heat inactivated	Gibco	26050088

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
MNI-glutamate	Tocris Bioscience	1490
(+)-Sodium L-ascorbate (Ascorbic acid)	Sigma-Aldrich	A4034
Insulin	Sigma-Aldrich	I5500
D-glucose	Sigma-Aldrich	G8270
HEPES	Sigma-Aldrich	H3375
TTX citrate	Tocris Bioscience	1069
L-glutamine	Gibco	25030081
Experimental models: Organisms/strains		
Mouse: C57BL/6J	The Jackson Laboratory	Stock No: 000664
Recombinant DNA		
pEGFP-N1	Clontech Laboratories	N/A
Software and algorithms		
ImageJ	National Institutes of Health	https://imagej.nih.gov/ij/
Other		
6-well Falcon Polystyrene Microplates	Fisher Scientific	351146
Compact stereo microscope	Olympus	SZ51
Dissecting tools- Forceps	Fine Science Tools	11210-10
Dissecting tools- Surgical Scissors	Fine Science Tools	14002-14
Dissecting tools- Double edge blade	Electron Microscopy Sciences	72000WA
Dissecting tools- Dental Scoop	Henry Schein	1008645
Vacuum filter system	Corning	431097
Tissue culture inserts	Merck Millipore	PICM0RG50
Tissue culture incubator	Thermo Scientific	Cat #: 50116047
Tissue slicer	Stoelting	51425
Helios Gene Gun	Bio-Rad Laboratories	1652431
Microscope slides – FisherBrand	Fisher Scientific	12-550-15
LaserCheck	Coherent	1098293
Gold wire (0.5mm dia, 99.99%)	Alfa Aesar	10966
Two-photon imaging microscope	Various	N/A
Two ultrafast femtosecond lasers	Various	N/A
Perfusion system with aeration and temperature control	Various	N/A

MATERIALS AND EQUIPMENT

Organotypic culture system and gene transfection

Tissue culture inserts (e.g., Millipore PICM0RG50)

Tissue culture incubator (35°C / 5% CO_2)

Compact stereo microscope (e.g., Olympus SZ51)

Tissue Slicer (e.g., Stoelting 51425)

Biolistic particle delivery system (e.g., Helios Gene Gun, Bio-Rad Laboratories 1652431)

MEM Eagle medium (Sigma-Aldrich M4642)

Phenol red solution (0.5%, Sigma-Aldrich P0290)

Two-photon imaging rig

Two-photon imaging microscope equipped with electro-optical modulators (e.g., Conoptics 350-80LA / BK / 302RM) or the equivalent for rapid (microseconds) control of laser power

Note: For more information about our specific configuration see Stein et al. (2019).

Two ultra-fast femtosecond lasers (e.g., Spectra-Physics Mai Tai; 930 nm imaging, 720 nm uncaging)

Perfusion system with aeration (95% O2 / 5% CO2) (e.g., Watson-Marlow SciQ 400 peristaltic pump)

Temperature controller for perfusion system (e.g., Warner Instrument TC-324C)

Imaging analysis system

Computer workstation and software for image analysis (e.g., ImageJ, NIH)

Artificial Cerebrospinal Fluid (aCSF)		
Reagent	Final concentration	Amount
NaCl	127 mM	7.42 g
$NaHCO_3$	25 mM	2.1 g
NaH_2PO_4	1.2 mM	0.173 g
KCl	2.5 mM	0.187 g
D-glucose	25 mM	4.5 g
$CaCl_2$ (1M)	0.3 or 2 mM	0.3 or 2 mL
ddH_2O	n/a	Adjust to 1 L
Total		**1 L**

⚠ CRITICAL: Adjust the pH (~7.2-7.4) by bubbling with carbogen (95% O_2 / 5% CO_2). Osmolarity should be 310 mOsm/L. Store aCSF at 4°C for up to 7 days.

Dissection media		
Reagent	Final concentration	Amount
Sucrose	234 mM	40 g
$CaCl_2$ (1M)	1 mM	0.5 mL
$MgCl_2$ (1M)	5 mM	2.5 mL
KCl	4 mM	0.15 g
D-glucose	10 mM	0.9 g
Sodium bicarbonate	26 mM	1.09 g
Phenol red solution (0.5%)	0.001%	1 mL
ddH_2O	n/a	adjust to 500 mL
Total		**500 mL**

⚠ CRITICAL: Dissection media must be filter sterilized (0.22 μm PES filter). Store dissection media at 4°C for up to 2 weeks. Before use, adjust the pH by bubbling with carbogen (95% O_2 / 5% CO_2).

Slice Culture Media (SCM)		
Reagent	Final concentration	Amount
MEM Eagle medium	8.4 g/L	8.4 g
Horse serum	20%	200 mL
$CaCl_2$ (1M)	1 mM	1 mL
$MgSO_4$ (1M)	2 mM	2 mL
L-Glutamine (200 mM)	1 mM	5 mL
Ascorbic acid (25%)	0.00125%	50 μL
Insulin (1 mg/mL)	1 mg/L	1 mL

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Reagent	Final concentration	Amount
D-glucose	13 mM	2.32 g
Sodium bicarbonate	5.2 mM	0.44 g
HEPES	30 mM	7.16 g
ddH_2O	n/a	adjust to 1 L
Total		1 L

⚠ CRITICAL: Add reagents to 750 mL ddH_2O in a 1 L beaker. Adjust the pH to 7.28 with 1N NaOH and the osmolarity to 320 mOsm with ddH_2O. SCM must be filter sterilized (0.22 μm PES filter). Store SCM at 4°C for up to 2 weeks. Warm SCM to 35°C before use.

STEP-BY-STEP METHOD DETAILS

Selection of pyramidal neurons

Timing: 5–15 min

The aim of this step is to select healthy CA1 pyramidal neurons with robust EGFP expression and characteristic depth from the slice surface for uniform imaging and uncaging quality.

1. Initial screening for EGFP-transfected CA1 neurons using epifluorescence.
   a. Transfer tissue culture inserts and SCM from six-well plates into 35 mm petri dishes.
   b. Place uncovered 35 mm petri dish under the low magnification objective (10×) and screen for EGFP transfected CA1 neurons.
   c. Mark the bottom of the petri dishes to indicate slices that have CA1 neurons with intact long apical dendrites.
   d. Return petri dish to the cell culture incubator.
2. Start the perfusion system.
   a. Directly prior to imaging, dissolve 10 mg of MNI-caged glutamate (Tocris, 1490) by adding aCSF (1 mL) directly to the vial and rock or shake for 2–5 min until powder is completely dissolved. As MNI-glutamate can be uncaged by white fluorescent light, protect as much as possible from light exposure. We find that the stock solution can be frozen once at −20°C without noticeable decrease in efficacy.
   b. Load the recirculating perfusion system and imaging chamber with aCSF containing 2.5 mM MNI-caged glutamate and 1 μM TTX. We use 6 mL total volume for our perfusion system. To decrease evaporation, we bubble the carbogen through water prior to bubbling the aCSF bath. In addition, we exchange the bath solution at minimum every 2.5 h.
   c. Turn on the heating system and set the temperature at 30°C.

   Note: MNI-caged glutamate is light sensitive. Avoid exposure to bright light in the solution preparation room or the imaging room.

3. When the temperature is stable, take one petri dish from the cell culture incubator. Using a scalpel blade, cut the culture membrane around the hippocampal slice in the form of a rectangle (Figures 3A and 3B).

   Note: Be sure to leave extra membrane around the slice to use for stabilizing the slice at the bottom of the imaging chamber (step 5).

4. Using fine tweezers, grab one corner of the membrane containing the hippocampal slice (Figure 3C) and place it into the imaging chamber with recirculating aCSF.

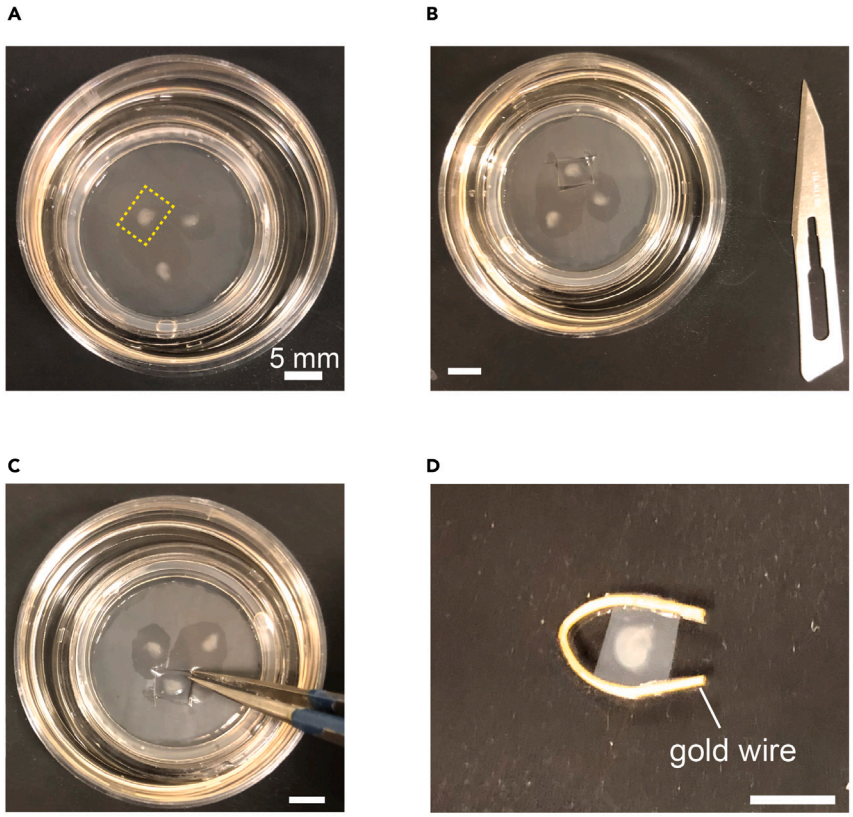

Figure 3. Transfer the hippocampal slices from culture inserts to imaging chamber

(A) Image of the culture inserted with hippocampal slices. Yellow box indicates the cutting area of the membrane of culture insert.

(B) Image showing after the membrane is cut on all four sides around one slice using a scalpel blade.

(C) Lift one corner and grab the membrane with an attached hippocampal slice with the tweezer.

(D) Put the gold wire on the membrane around the slice to hold in the imaging chamber.

5. Using a bent gold wire (Alfa Aesar, 10966) anchor to weigh down membrane and prevent slice movement in the imaging chamber (Figure 3D).
6. Maintain the temperature of the aCSF within 29°C–31°C.
7. Locate EGFP-expressing CA1 pyramidal neurons under low magnification objective lens (10×) and then move to high magnification objective (e.g., Olympus, 60× IR, NA 1.0, WD 2.0 mm).
8. Using two-photon imaging with the 930 nm laser, select healthy EGFP-expressing CA1 pyramidal neurons using the criteria listed below.
   a. CA1 pyramidal neuron morphology with straight, extended apical dendrite (Figure 4A)
   b. Intact apical and basal dendrites with clear secondary and tertiary dendrites (Figure 4A), as some cells will have dendrites cut off during slice preparation (Figure 4B)
   c. Dendritic diameter is smoothly tapered (Figure 4C) without blebbing (Figure 4B and 4C)

Selection of dendritic regions of interest (ROIs) for imaging

Timing: 10–15 min

This step describes the guidelines to select dendritic segments and target spines

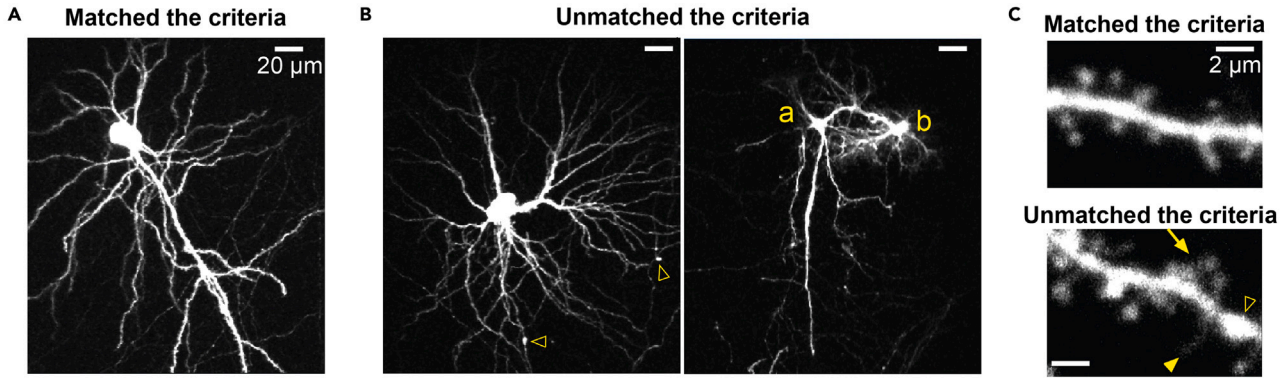

Figure 4. Selection of EGFP-transfected hippocampal CA1 neurons

(A) Image of CA1 pyramidal neuron matching the criteria for selection for experiments.

(B) Images of cells that do not match the criteria for selection for experiments. Left: CA1 neuron with apical dendrite cut off during dissection. Open arrow heads mark blebs showing unhealthy basal dendrites. Right: Unhealthy neuron (a) and non-neuronal cell (b) fused.

(C) Images of dendrites that matched criteria (upper) and do not match criteria (bottom) for selection for experiments. Open arrowhead marks the bleb. Filled arrowhead and arrow mark the filopodium and fuzzy membrane, respectively.

9. Select secondary or tertiary basal dendrites located 40–80 μm from the soma and 15–45 μm below the slice surface. Others have successfully implemented this protocol at apical dendrites (Albarran et al., 2021).

10. To prevent photobleaching and phototoxicity during screening dendrites, use low intensity laser power (~ 1 mW at the sample) and higher-speed imaging mode (e.g., 256 × 256 pixels, 0.14 μm/pixel, 500 msec/frame).

⚠ CRITICAL: Criteria to select target dendritic segments (Figure 4C).

   a. Dendritic segments do not include blebbing and have similar fluorescence intensity and diameter in ROIs.
   b. Dendritic segments include multiple well-isolated spines (> 2 μm) without filopodia and fuzzy membrane.
   c. The target dendritic segments include multiple spines which have clearly separated spine heads from the dendrites.

11. Select dendrites that have typical spine density and contain a well-isolated spine (> 2 μm from neighboring spines) to use for input-specific stimulation. The target spine should be average or modestly larger than average compared to the other spines in the ROI (Figures 5A and 5B). Avoid selecting stubby-type spines (Figure 5B).

Note: A typical dendritic ROI includes one target spine and 3–6 neighboring spines that match criteria for inclusion.

Time-lapse imaging and induction of spine shrinkage

Timing: [1–2 h]

12. Place the slice in the recirculating aCSF at least 15 min before imaging and uncaging to equilibrate the slice into the aCSF.
13. Choose a low frequency uncaging (LFU) protocol. We have provided two LFU protocols, both in 0 mM Mg^{2+} aCSF, one with 0.3 mM Ca^{2+} and the other with 2 mM Ca^{2+}.

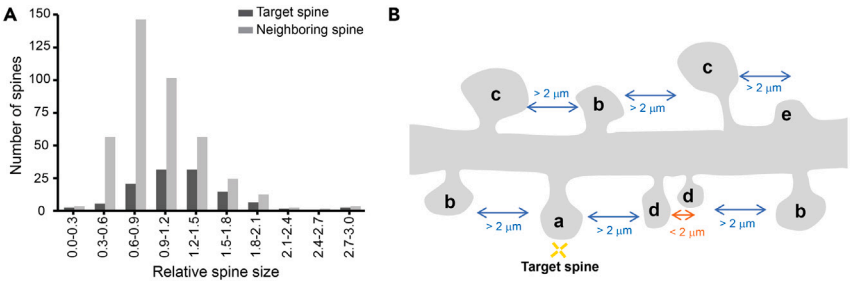

Figure 5. Selection of target spine for stimulation

(A) Histogram of relative spine size of target spines (dark grey, n=108) and neighboring spines (light grey, n=400) from selected dendritic segments (n=93). Data included is both new data and published data from Oh et al. (2013).

(B) The diagram shows how to select a target spine (yellow cross). Select a target spine matched with the criteria, ideally located in the center area of ROI. Spines in categories a and b are suitable target spines, categories c-e should be avoided. a: Spine is well-isolated from neighboring spines (> 2 μm) and average size with neck. b: Spines which are also well-isolated from neighboring spines with average size. c: Well-isolated spines but size is bigger than average. d: Spines are too close to each other (< 2 μm) and are smaller than average. e: Spine with 'stubby' morphology.

a. Glutamate uncaging protocol for 0.3 mM $Ca^{2+}$and 0 mM Mg^{2+} aCSF
   i. Two-photon uncaging laser set to 720 nm and 6–8 mW at the sample
   ii. 90 pulses of 1 ms pulse duration at 0.1 Hz
b. Glutamate uncaging protocol for 2 mM $Ca^{2+}$and 0 mM Mg^{2+} aCSF
   i. Two-photon uncaging laser set to 720 nm and 6–8 mW at the sample
   ii. 90 pulses of 0.2 ms pulse duration at 0.1 Hz

14. For imaging, set the laser to 930 nm and 1–1.2 mW at the sample. Take the baseline z-stack of the ROI with 1 μm z-steps at −5 min and 0 min before uncaging. Typically, 5–8 z-steps are sufficient to cover the dendritic segment.

⚠ CRITICAL: If spine fluorescence intensity from −5 min and 0 min baseline images changes by more than 20%, select a different ROI and target spine.

15. Deliver glutamate uncaging laser pulses to a target spine by parking the laser beam at a point ~ 0.5 μm from the spine head in the direction away from the dendrite.

⚠ CRITICAL: Refocus and adjust laser beam parking spots between pulses.

16. Take a z-stack of the ROI with 1 μm z-steps every 5 min after uncaging until 40 min.

Note: Inter-imaging interval and duration of maximum imaging time can be varied depending on experiment purpose. When the imaging interval is decreased, it is recommended to use lower imaging laser power to minimize photobleaching.

⚠ CRITICAL: Because the health of hippocampal slices deteriorates over time after being placed in the imaging chamber, it is typical to change to a new slice after 2–2.5 h and to refresh the recirculating aCSF with TTX and MNI-caged glutamate after 2–3 h. We recommend recording a single target spine per cell and one cell per slice to reduce slice health issues.

Quantification of spine size

Timing: [1–2 h]

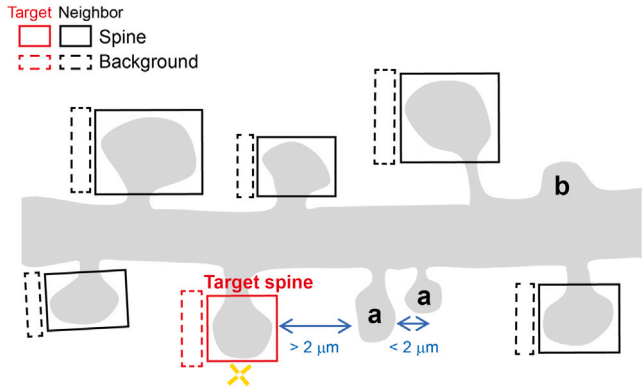

Figure 6. Image analysis

The approach for selecting spine and background ROIs for quantification of fluorescence intensities in spines. Red solid box (stimulated target spine) and black solid box (neighboring spines) surround the spine heads in a single Z stack image which is the brightest. Adjacent background is marked by dashed red and black boxes. (a) and (b) are excluded from analysis because (a) spines are too close from next spines (< 2 μm) and (b) spine is not clearly separated from dendrite.

17. Draw a box to select a region of interest (ROI) surrounding the stimulated and unstimulated spines on the imaged dendritic segment. Measure integrated fluorescence intensity from the brightest slice in the z stack for each spine at each time point (Figure 6).

    Note: Choose only spines that are well-separated from the dendrite to avoid contamination from the dendritic signal.

18. Select a background ROI box spanning the same distance from the dendrite as the spine head ROI to ensure an equivalent background signal. Measure the mean fluorescence intensity from these ROIs. Multiply this number by the number of pixels in the spine head ROI and subtract this result from the integrated spine fluorescence to generate background-subtracted spine fluorescence intensity, as described (Woods et al., 2011).
19. Normalize the background-subtracted spine fluorescence intensity signal over time by dividing the signal at each time point after stimulation with the average background-subtracted spine fluorescence intensity signal from the time points before stimulation. This is the measure that we report as "Estimated Spine Volume" in our papers.

    Note: Spine brightness measurements give an accurate estimate of relative spine volume when compared with electron microscopy (Holtmaat et al., 2005).

20. Relative spine size (Figure 5A) was calculated using the background-subtracted, integrated fluorescence intensity (average of the −5 and 0 time points) of an individual spine and dividing that by the average background-subtracted, integrated fluorescence intensity (average of the −5 and 0 time points) of all measurable neighboring spines on the same dendritic ROI.

EXPECTED OUTCOMES

In optimal conditions, this low-frequency glutamate uncaging protocol results in shrinkage of ~70% of spines to < 85% from their baseline size (Figure 7). In conjunction with molecular genetic and pharmacological manipulations, this protocol can be used to delineate critical components in the molecular cascade for dendritic spine shrinkage by comparing spine shrinkage in the same neurons between different conditions, for example the competitive NMDA receptor (NMDAR) antagonist CPP blocks spine shrinkage induced by LFU (90 pulses at 0.1 Hz) (Figure 7).

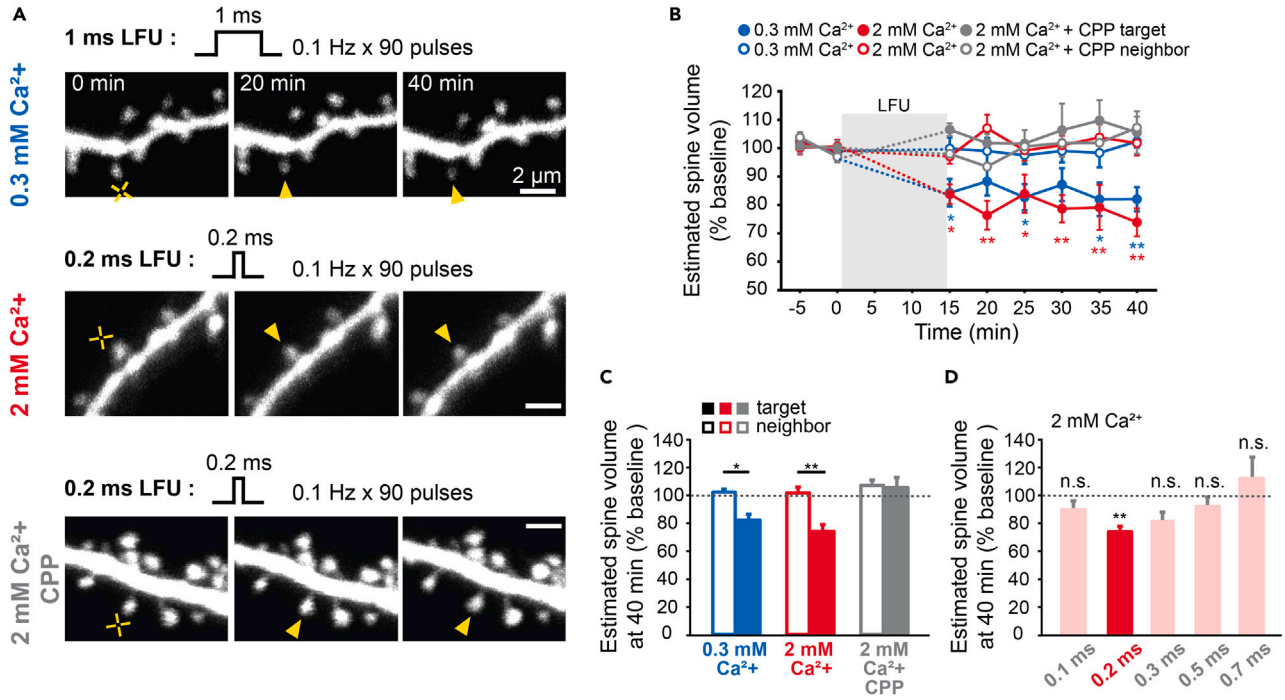

Figure 7. Summary of stimulation protocols to induce input-specific spine shrinkage

(A) Images of dendrites from EGFP-transfected hippocampal CA1 neurons before and after glutamate uncaging stimulation. Stimulation protocols are diagrammed above the images.

(B and C) Quantification of spine size over time reveals significant and long-lasting spine shrinkage that is blocked by inhibiting the NMDA receptor with 10 µM CPP.

(D) Spine shrinkage at 40 min after uncaging stimuli of different pulse durations in 2 mM Ca^{2+}.

** p<0.01, * p<0.05, *n.s.* p>0.5. N values (spines/cells): 0.3 mM Ca^{2+} - 1 ms (7/4); 2 mM Ca^{2+} - 0.1 ms (7/ 5), 0.2 ms (9/8), 0.3 ms (10/7), 0.5 ms (7/ 6), 0.7 ms (6/6), 0.2 ms + CPP (7/4). Error bars represent standard error of the mean.

QUANTIFICATION AND STATISTICAL ANALYSIS

To reduce variability originating from individual animals and different dissections, at least 3 independent preparations should be included per each condition, and multiple conditions should be assessed from each preparation. Spines should be excluded from analysis if their size fluctuates more than ± 20% during baseline imaging (−5 and 0 min time points). After ensuring that our laser power is set appropriately, we exclude from the analysis dendrites that show fluorescence intensity decreases during imaging by photobleaching, as we find that it is a sign of unhealthy cells.

To generate a line graph, as in Figure 7B, we calculate the mean and standard error of the mean (SEM) from estimated spine sizes (%) of each stimulation condition. Results from six to ten spines per condition (one target spine per cell) are usually enough to reach statistical significance. The estimated sizes of all analyzable neighboring spines from a dendritic segment are averaged together and represented as a single point. To quantify spine size changes relative to baseline in Figure 7B, we use two-way repeated-measure ANOVA with Dunnett's post hoc. To compare spine size at 40 min after stimulation between the target (stimulated spines) and neighbors (unstimulated spines) across multiple conditions in Figure 7C, we use two-way ANOVA with Tukey's multiple comparison test. We use Prism software for statistical analysis, and significance is set at $p < 0.05$ for ANOVA test.

LIMITATIONS

The stimulation protocols presented here use 0 mM Mg^{2+} to remove Mg^{2+} from NMDA receptors, which is required for induction of synaptic plasticity and is usually accomplished through a whole-cell

patch clamp recording configuration. However, whole-cell patch clamp recording causes dialysis of the intracellular contents into the patch pipette, resulting in a dramatic decline in spine structural plasticity, likely due to loss of actin monomers from the cell into the pipette (Matsuzaki et al., 2004). If the experimental question necessitates physiological concentration of Mg^{2+} (~ 1 mM), this protocol will need to be adapted by incorporating other methods to remove Mg^{2+} from NMDA receptors during glutamate uncaging (e.g., depolarization of the cell in perforated patch clamp configuration).

We have tested and successfully induced input-specific spine shrinkage using these protocols in organotypic cultured hippocampal slices at DIV 11–15 from mice (C57BL/6J) and rats (Sprague-Dawley) and acute hippocampal slices from 17–21 day old mice (C57BL/6J). It is possible and even likely that this protocol will need to be adjusted for different strains and different ages.

We have used primarily spines of average or modestly larger than average sizes. We chose this range because smaller than average sized spines are often unstable and prone to shrink and eliminate independent of stimulation. Furthermore, we find that the largest spines (~ top 5%) are exceptionally stable and can be resistant to shrinkage induction using the described protocols.

TROUBLESHOOTING

Problem 1

Induction of spine shrinkage fails (steps 15 and 16)

Failure to induce spine shrinkage could result from several possible issues. First, the cells could be unhealthy. Second, imaging and uncaging conditions are not optimized.

Potential solution 1

During the preparation time for imaging, sometimes the health of the neuron can decline. Try again with another neuron.

Potential solution 2

Revisit the beads to check alignment of imaging and uncaging lasers. Alternatively, tune the uncaging laser to 930 nm and check the image alignment between both lasers.

Confirm that the uncaging laser is focused at ~ 0.5 μm from the spine head.

Be sure to re-focus directly preceding *every* stimulation pulse.

Check stability of the bath aCSF temperature – fluctuating temperature can be problematic and causes drift.

Potential solution 3

Increased and decreased laser power can cause spine shrinkage failure. Re-check that the uncaging laser (720 nm) power is 6–7 mW at the sample.

Potential solution 4

After checking Potential Solution 1–3, if spine shrinkage continues to fail, test high frequency uncaging (HFU) protocol to induce spine enlargement (Stein et al., 2015).

Set the uncaging laser (720 nm) to 7–11 mW at the sample.

Apply HFU protocol (60 pulses of 2 ms pulse duration at 2 Hz in 2 mM Ca^{2+} and 0 mM Mg^{2+} aCSF) to the spine on different dendrites of the same cell.

If the stimulated spine shows transient spine enlargement immediately following stimulation, the target cell and dendritic spines are healthy and the uncaging system is properly aligned. Select a spine on another dendrite of the same cell, decrease laser power by 10–20%, and re-try LFU.

If the stimulated spine does not show transient spine enlargement by HFU, increase laser power up to ~12 mW at the sample and re-try inducing spine enlargement with HFU. If still no spine enlargement, the preparation may be poor – try another culture preparation.

Problem 2
Photobleaching of the sample after photostimulation (step 16)

Potential solution 1
Minimize the intensity of the imaging laser (930 nm) during focusing between photostimulation events to under 1 mW.

Potential solution 2
Check laser alignment. The uncaging laser beam must not directly hit the target spine. It will cause photobleaching and can damage the membrane of the spine and dendrite.

Problem 3
Photobleaching of the sample during baseline imaging (step 14)

Potential solution 1
Reduce the intensity of the imaging laser (930 nm). If imaging laser intensity needs to be higher than 1.5–2 mW to see clear spine shape, check depth of the cell and target dendrite.

Potential solution 2
Check target cell health. During baseline imaging, cell health can be changed. Check the target cell morphology and if dendrites have blebs or more filopodia and fuzzy membranes, try with other neurons.

Problem 4
Weak fluorescence intensity of sample (steps 9–12)

Potential solution 1
Increase the amount of plasmid encoding fluorescent protein on the bullets for biolistic transfection. We typically use 10–15 μg EGFP to coat 8 mg gold particles. Increase as needed up to 40 μg. Don't increase to more than 40 μg or the bullets may clump together resulting in poor transfection.

Potential solution 2
Transfect the neurons longer prior to imaging in order to increase EGFP expression levels.

Problem 5
Uncaging laser is burning the tissue (step 15)

Potential solution 1
Re-check that the uncaging laser (720 nm) power is 6–7 mW at the sample.

Potential solution 2
Check the laser alignment. The uncaging laser beam must not directly hit the spines and dendrites of the target neuron or it will cause photobleaching and can damage the membrane of the spine and dendrite.

Potential solution 3

If Potential solution 1 and 2 are not successful, reduce the power of the uncaging laser.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Karen Zito, kzito@ucdavis.edu.

Materials availability

All materials used in this protocol are commercially available.

Data and code availability

The datasets supporting the current study have not been deposited in a public repository, but are available from the corresponding author on request.

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AUTHOR CONTRIBUTIONS

J.J. developed the updated protocol in 2 mM Ca^{2+}, performed experiments, and wrote the manuscript. W.C.O. developed the original protocol in 0.3 mM Ca^{2+}, performed experiments, and edited the manuscript. M.A. performed experiments and edited the manuscript. K.Z. provided input on protocol development and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Albarran, E., Raissi, A., Jaidar, O., Shatz, C.J., and Ding, J.B. (2021). Enhancing motor learning by increasing the stability of newly formed dendritic spines in the motor cortex. Neuron *109*, 3298–3311.

Holtmaat, A.J., Trachtenberg, J.T., Wilbrecht, L., Shepherd, G.M., Zhang, X., Knott, G.W., and Svoboda, K. (2005). Transient and persistent dendritic spines in the neocortex in vivo. Neuron *45*, 279–291.

Matsuzaki, M., Honkura, N., Ellis-Davies, G.C.R., and Kasai, H. (2004). Structural basis of long-term potentiation in single dendritic spines. Nature *429*, 761–766.

Oh, W.C., Hill, T.C., and Zito, K. (2013). Synapse-specific and size-dependent mechanisms of spine structural plasticity accompanying synaptic weakening. Proc. Nat. Acad. Sci. U S A *110*, E305–E312.

Opitz-Araya, X., and Barria, A. (2011). Organotypic hippocampal slice cultures. J. Vis. Exp. *48*, 55359.

Stein, I.S., Gray, J.A., and Zito, K. (2015). Non-Ionotropic NMDA receptor signaling drives activity-induced dendritic spine shrinkage. J. Neurosci. *35*, 12303–12308.

Stein, I.S., Hill, T.C., Oh, W.C., Parajuli, L.K., and Zito, K. (2019). Probing the structural and functional plasticity of dendritic spines using two-photon glutamate uncaging. In Multiphoton Microscopy, E. Hartvelt, ed. (Springer Nature), pp. 65–85.

Stein, I.S., Park, D.K., Flores, J.C., Jahncke, J.N., and Zito, K. (2020). Molecular mechanisms of non-ionotropic NMDA receptor signaling in dendritic spine shrinkage. J. Neurosci. *40*, 3741–3750.

Stein, I.S., Park, D.K., Claiborne, N., and Zito, K. (2021). Non-ionotropic NMDA receptor signaling gates bidirectional structural plasticity of dendritic spines. Cell Rep. *34*, 108664.

Woods, G.F., Oh, W.C., Boudewyn, L.C., Mikula, S.K., and Zito, K. (2011). Loss of PSD-95 Enrichment Is Not a Prerequisite for Spine Retraction. J. Neurosci. *31*, 12129–12138.

Woods, G., and Zito, K. (2008). Preparation of gene gun bullets and biolistic transfection of neurons in slice culture. J. Vis. Exp. *12*, e675.