STAR Protocols

Protocol

Light and electron microscopic imaging of synaptic vesicle endocytosis at mouse hippocampal cultures

Part I Preparation of hippocampal neuron

P0-1 pup Dissection | Plating at DIV0 | Transfection at DIV7 | Imaging at DIV14

14 days

1 day | **8 days**

Part II Acquisition of fluorescence image
60 x
Bouton fluorescence

Part III Acquisition of EM image
EM
Bouton image

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Highlights

Detailed protocol for primary culture and transfection of mouse hippocampal neurons

Light microscopy and analysis of endocytosis in cultured neurons

Electron microscopy and analysis of vesicle and endosome formation

Following the release of neurotransmitters at synaptic vesicles via exocytosis, endocytosis is initiated to retrieve vesicles that have fused with the plasma membrane of nerve terminals and recycle them, thus sustaining synaptic transmission. Here, we describe imaging-based protocols for quantitative measurements of endocytosis at cultured synapses. These protocols include (1) primary culture of mouse hippocampal neurons, (2) studying endocytosis at neurons transfected with a pH-sensitive synaptophysin-pHluorin2× using fluorescent microscopy, and (3) imaging endocytosis at fixed neurons with electron microscopy.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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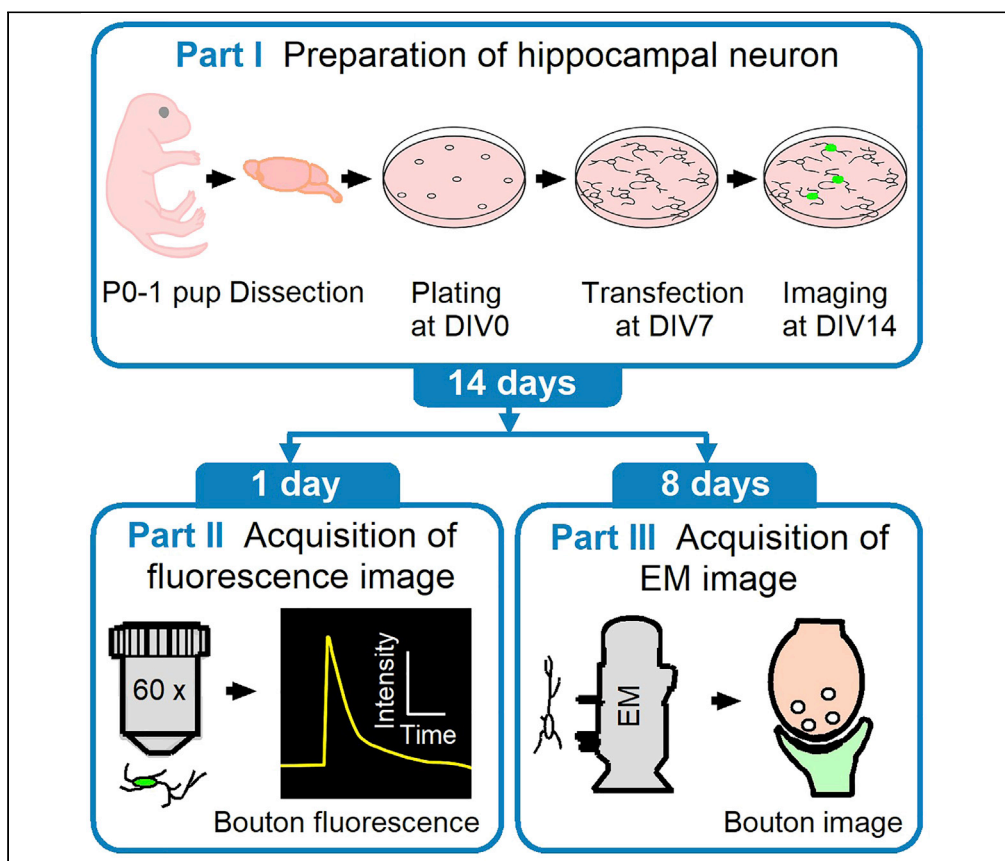## Protocol

Light and electron microscopic imaging of synaptic vesicle endocytosis at mouse hippocampal cultures

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SUMMARY

Following the release of neurotransmitters at synaptic vesicles via exocytosis, endocytosis is initiated to retrieve vesicles that have fused with the plasma membrane of nerve terminals and recycle them, thus sustaining synaptic transmission. Here, we describe imaging-based protocols for quantitative measurements of endocytosis at cultured synapses. These protocols include (1) primary culture of mouse hippocampal neurons, (2) studying endocytosis at neurons transfected with a pH-sensitive synaptophysin-pHluorin2× using fluorescent microscopy, and (3) imaging endocytosis at fixed neurons with electron microscopy.

For complete details on the use and execution of this protocol, please refer to Wu et al. (2016) and Wu et al. (2021).

BEFORE YOU BEGIN

By recycling vesicles, endocytosis sustains synaptic transmission and helps to relieve short-term synaptic depression (Sankaranarayanan and Ryan, 2000; Granseth et al., 2006; Wienisch and Klingauf, 2006; Balaji and Ryan, 2007; Sun et al., 2010; Leitz and Kavalali, 2011; Kavalali and Jorgensen, 2014; Wu et al., 2014a, 2014b; Kononenko and Haucke, 2015; Chanaday and Kavalali, 2018; Gan and Watanabe, 2018; Chanaday et al., 2019; Shin et al., 2021). Impairments of endocytosis may contribute to the generation of neurological disorders, such as neurodegeneration and spinocerebellar ataxia 13 (Saheki and De Camilli, 2012; Busch et al., 2014; Xu et al., 2016; Wu et al., 2021). Study of endocytosis is thus crucial to understanding synaptic transmission, synaptic plasticity, and neurological disorders. To achieve these goals, it is necessary to have reliable and mature tools for exploring the molecular mechanisms underlying endocytosis as well as related diseases. Here, we provide a reliable experimental protocol for the measurement of endocytosis at hippocampal synapses cultured from newborn mice.

Institutional permissions

All animal study protocols were approved by the Animal Care and Use Committee (ACUC) at the National Institute of Neurological Disorders and Stroke (NINDS), Bethesda, USA. All procedures were conducted under the guidelines made by the ACUC. The protocol numbers for the animal study are NINDS ASP 1170-22 and NINDS ASP 1259-21.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
3,3′-Diaminobenzidine	Sigma	Cat#D8001
AP5	Tocris	Cat#3693
Agarose	Bioline	Cat#Bio-41025
Albumin Bovine Serum	Sigma	Cat#A2153
B-27 supplement (50×)	Thermo Fisher Scientific	Cat#17504044
$CaCl_2$	Quality Biological	Cat#351-130-721
CNQX	Tocris	Cat#0190
Cytosine β-D-arabinofuranoside (Ara-C)	Sigma	Cat#C6645
DMEM	Gibco	Cat#11995040
DNase I	Worthington	Cat#LK003170
Fetal Bovine Serum (FBS, heat inactivated)	Thermo Fisher Scientific	Cat#10082147
Glucose	Sigma	Cat#G8270
GlutaMAX™ supplement	Thermo Fisher Scientific	Cat#35050061
Glutaraldehyde aqueous solution (70%)	Emsdiasum	Cat#16365
HBSS ([-] Ca^{2+}, [-] Mg^{2+})	Thermo Fisher Scientific	Cat#14175095
HBSS ([+] Ca^{2+}, [+] Mg^{2+})	Thermo Fisher Scientific	Cat#14025134
HEPES	Sigma	Cat#H3375
HEPES solution (X100)	Thermo Fisher Scientific	Cat#15630080
High-vacuum silicone grease	Sigma	Cat#Z273554-1EA
Horseradish peroxidase (HRP)	Sigma	Cat#P8125
Hydrogen peroxide solution	Sigma	Cat#216763
Immersion oil type F	Nikon	Cat#MXA22168
Insulin from bovine pancreas	Sigma	Cat#I5500
Lead citrate	Emsdiasum	Cat#22410
Lens cleaner	Purosol	www.purosol.com
Lipofectamine™ LTX reagent with PLUS™ reagent	Thermo Fisher Scientific	Cat#15338030
Magnesium chloride solution ($MgCl_2$)	Quality Biological	Cat#351-033-721
Neurobasal-A medium	Thermo Fisher Scientific	Cat#10888022
Opti-MEM™ I reduced serum medium	Thermo Fisher Scientific	Cat#31985070
Papain	Worthington	Cat#LK003176
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat#15140122
Pierce™ horseradish peroxidase	Thermo Fisher Scientific	Cat#31491
Poly-L-lysine solution	Sigma	Cat#P4832
Potassium chloride (KCl)	Sigma	Cat#P4504
Osmium tetroxide (OsO_4)	Emsdiasum	Cat#19160
Ovomucoid inhibitor	Worthington	Cat#LK003182
Sodium Acetate buffer	Sigma	Cat#S7899
Sodium cacodylate	Emsdiasum	Cat#12310
Sodium chloride (NaCl)	Sigma	Cat#S7653
Sodium phosphate (Na_2HPO_4)	Sigma	Cat#S9763
SPI-Pon™ 812 Embedding Kit	Structure Probe Inc.	Cat#02660-AB
SYBR™ Safe DNA gel stain	Thermo Fisher Scientific	Cat#S33102
Trypsin inhibitor from chicken egg white	Sigma	Cat#T9253-5G
Uranyl Acetate	Emsdiasum	Cat#22400-2
Experimental models: Organisms/strains		
C57BL/6J mice (P0–1, male or female pups)	The Jackson Laboratory	Cat#000664
Recombinant DNA		
pcDNA3-SypHluorin 2× (S2×)	(Zhu, et al., 2009)	Addgene 37004
Software and algorithms		
Fiji	(Schindelin, et al., 2012)	https://imagej.net/Fiji/Downloads
Igor	WaveMetrics	https://www.wavemetrics.com/

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Microsoft Excel	Microsoft	https://www.microsoft.com
NIS-Elements AR 5.20.02	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements
Other		
60× Oil Objective	Nikon	Apo VC
CCD Camera (for SypH imaging)	Andor Technology	iXon X3 897
CCD Camera (for EM imaging)	AMT	XR-100
Chamber platform	Warner	Cat#P2/PH2
Chamber with field stimulation	Warner	Cat#RC-21BRFS
Transmission Electron Microscope	JEOL	JEM-200 CX
Fluorescence Microscope	Nikon	Eclipse Ti-E
Laminin coated coverslip (25 mm diameter)	Neuvitro	Cat#GG-25-Laminin
Isolated Pulse Stimulator	A-M Systems	Model 2100
Millex-GP syringe filter unit (0.22 μm)	MilliporeSigma	SLGP033RB
Scissors (angled)	WPI	Cat#500047
Scissors (fine)	WPI	Cat#500086
Stimulus Isolation Unit	Warner	SIU-102
Tweezers (blunt)	WPI	Cat#500336
Tweezers (curved)	WPI	Cat#14097
Tweezers (fine)	WPI	Cat#500233
Ultramicrotome	Leica	Ultracut S

MATERIALS AND EQUIPMENT

The following solutions and media will be prepared for culturing hippocampal neurons from 5 pups and for the following imaging experiments. Information about the stuff used in the protocol can be found in the key resources table.

Dissection solution

Reagent	Final concentration	Volume (mL)
HEPES (100×)	1×	0.5
Penicillin/Streptomycin (100×)	1×	0.5
Fetal Bovine Serum (FBS)	20%	10
HBSS ([-] Ca^{2+}, [-] Mg^{2+})	1×	39
Total	n/a	50

Note: It is used for brain dissection. Prepare it before dissection. Cool and store it on ice during dissection.

Papain solution

Reagent	Final concentration	Amount
Papain	20−25 U/mL	100−125 U
DNase I	100−120 U/mL	500−600 U
HBSS ([+] Ca^{2+}, [+] Mg^{2+})	1×	5 mL
Total	n/a	5 mL

Note: Papain is a sulfhydryl protease used for cell dissociation. DNase I is a deoxyribonuclease used for digesting DNA that has leaked from damaged cells into the dissociation medium. Prepare the solution before brain dissection. Warm the HBSS in a 37°C water bath for 10 min; add papain and DNase to warm HBSS. Filter the solution with a 0.22 μm syringe filter and store it in a 15-mL tube in a 37°C incubator before use.

Plating medium

Reagent	Final concentration	Volume
Fetal Bovine Serum (FBS)	10%	1 mL
B27 supplement (50×)	2%	200 μL
Insulin (10 mg/mL)	10 μg/mL	10 μL
Glutamax (100×)	1%	100 μL
Neurobasal-A	1×	8.68 mL
Total	n/a	10 mL

Note: Prepare 10 mg/mL insulin solution (adding drops of 1 N HCl to fully dissolve insulin in ddH$_2$O) and store at −20°C, best used within one year. The Plating Medium is used for plating cells on coverslips or bottom of plate. Prepare it before the dissection. Filter the medium with a 0.22 μm syringe filter and warm it in the 37°C incubator before use.

1/1 solution

Reagent	Final concentration	Amount
Ovomucoid inhibitor	2.5 mg/mL	12.5 mg
DNase I	100−120 U/mL	500−600 U
HBSS ([+] Ca^{2+}, [+] Mg^{2+})	1×	5 mL
Total	n/a	5 mL

Note: It is used to stop digestion induced by the Papain Solution. Ovomucoid inhibitor is a protease inhibitor. Prepare it after brain dissection. Filter the solution with a 0.22 μm syringe filter and store it at room temperature (22°C–24°C, RT) before use.

10/10 solution

Reagent	Final concentration	Amount
Trypsin inhibitor	10 mg/mL	100 mg
Albumin Bovine Serum	10 mg/mL	100 mg
HBSS ([+] Ca^{2+}, [+] Mg^{2+})	1×	10 mL
Total	n/a	10 mL

Note: It is used to trap dead cells and debris. Prepare it after brain dissection. Warm the solution in a 37°C water bath for 10 min to fully dissolve all solutes. Filter the solution with a 0.22 μm syringe filter and store at RT before use.

Feeding medium

Reagent	Final concentration	Volume
B27 supplement (50×)	2%	200 μL
Glutamax (100×)	1%	100 μL
Neurobasal-A	1×	9.7 mL
Total	n/a	10 mL

Note: It is used for medium changes during culture maintenance. Prepare it before changing the medium. Filter it with a 0.22 μm syringe filter and warm it in a 37°C incubator for 30 min before use.

Saline solution		
Reagent	Final concentration	Amount
NaCl	119 mM	1.3909 g
KCl	2.5 mM	0.0373 g
HEPES	25 mM	1.1916 g
Glucose	30 mM	1.0852 g
2 M CaCl$_2$ solution	2 mM	200 μL
1 M MgCl$_2$ solution	2 mM	400 μL
10 mM CNQX	10 nM	200 μL
50 mM AP5	50 nM	200 μL
ddH$_2$O	n/a	199 mL
Total	n/a	200 mL

Note: CNQX and AP5 are used to block postsynaptic activities, which have the potential to induce recurrent activity without additional stimulation. Prepare 10 mM CNQX and 50 mM AP5 solutions, store them at −20°C, best used within one year. The Saline Solution is used in the light imaging experiments. Prepare it, adjust pH to 7.2 with 1 N NaOH. Store at 4°C, best used within two weeks.

90 mM KCl solution		
Reagent	Final concentration	Amount
NaCl	31.5 mM	0.3682 g
KCl	90 mM	1.3419 g
HEPES	25 mM	1.1916 g
Glucose	30 mM	1.0852 g
2 M CaCl$_2$ solution	2 mM	200 μL
1 M MgCl$_2$ solution	2 mM	400 μL
10 mM CNQX	10 nM	200 μL
50 mM AP5	50 nM	200 μL
ddH$_2$O	n/a	199 mL
Total	n/a	200 mL

Note: It is used to depolarize the neurons and induce exocytosis and endocytosis in cultured neurons in EM experiments. Prepare it and adjust pH to 7.2 with 1 N NaOH. Store it at 4°C, best used within two weeks.

3,3′-Diaminobenzidine (DAB) solution		
Reagent	Final concentration	Amount
DAB	0.5 mg/mL	5 mg
30% H$_2$O$_2$ in H$_2$O	0.3%	100 μL
Filtered H$_2$O	n/a	10 mL
Total	n/a	10 mL

Note: It is used for the HRP staining in the EM experiments. Prepare it before experiment. Run the DAB Solution on the shaker overnight (12 h) to make sure the DAB dissolves completely in the water. Add the H$_2$O$_2$ and filter with a 0.22 μm syringe filter just before use.

⚠ CRITICAL: DAB is a suspected carcinogen; wear a lab coat, gloves, and protective goggles while working with it. Discard it appropriately after use.

Epoxy resin

Reagent	Final concentration	Volume (mL)
EPON812	48.3%	98
DDSA	16.3%	33
NMA	34.0%	69
DMP-30	1.5%	3
Total	n/a	203

Note: It is used for embedding and infiltration in the EM experiment. Reagents are from SPI-Pon^{TM} 812 Embedding Kit. Prepare it on the day of processing samples. Mixture made by volume. Mix well and store under vacuum to degas for 2 h, because bubbles, especially those invisible to the naked eye, can cause cavities in the resin during sectioning. Discard it after use.

STEP-BY-STEP METHOD DETAILS

Part I. Hippocampal neuron culture

High-quality cell culture is crucial for imaging experiments. Here, we detail a reliable and effective protocol for hippocampal neuron culture.

Hippocampal harvest

⏱ Timing: 30 min

1. Sterilize tools with 70% ethanol 30 min before dissection (Figure 1A).
2. Prepare the Dissection Solution, Papain Solution and Plating Medium.
3. Transfer 10–15 mL of ice-cold Dissection Solution to each of four 60-mm Petri dishes. The solution should be enough to cover the tissue in the dish. Keep dishes on ice.
4. Decapitate a P0–P1 pup with angled scissors and keep the head in the first dish (Figures 1B and 1C). Always keep a frozen-cold metal plate under the dish to keep the Dissection Solution cool.

   Note: 1) Both male and female pups will be used. 2) 3–4 metal plates should be precooled in a −20°C freezer for 2–3 h before dissection. The plate under the dish should be replaced with one precooled in the freezer every 10–15 min.

5. Remove the scalp with two tweezers.
6. Pin down the head by impaling the eyes with blunt tweezers; make a small incision at the midline between the eyes with the tip of another curved tweezers, then open the skull along the midline between the incision and the foramen magnum. Keep cuts shallow to avoid damaging the brain.
7. Using tweezers, remove the two halves of the skull (Figure 1D), separate the brain from the skull base, and transfer the isolated brain to the second dish (Figure 1E).
8. Under the dissection microscope (1×) (Figure 1F), hold the brainstem with blunt tweezers and remove cerebral hemisphere with fine tweezers (Figure 1G).
9. Gently peel off meninges surrounding the hippocampus with fine tweezers. The hippocampus, a C-shaped structure, should be clearly visible (Figure 1H). The concave side of the hippocampus, facing a ventricle, should be already free.
10. Use blunt tweezers to pin down the cerebral cortex and isolate the hippocampus along its convex side with fine tweezers. Using a cut 1-mL pipette tip, transfer the hippocampal tissue to the third dish (Figure 1I).
11. Use fine scissors to cut off the remaining dentate gyrus and subiculum around the hippocampus as much as possible; transfer the cleaned hippocampal tissue to the fourth dish.
12. Chop each hippocampus into 5–10 pieces with fine scissors (Figure 1J).
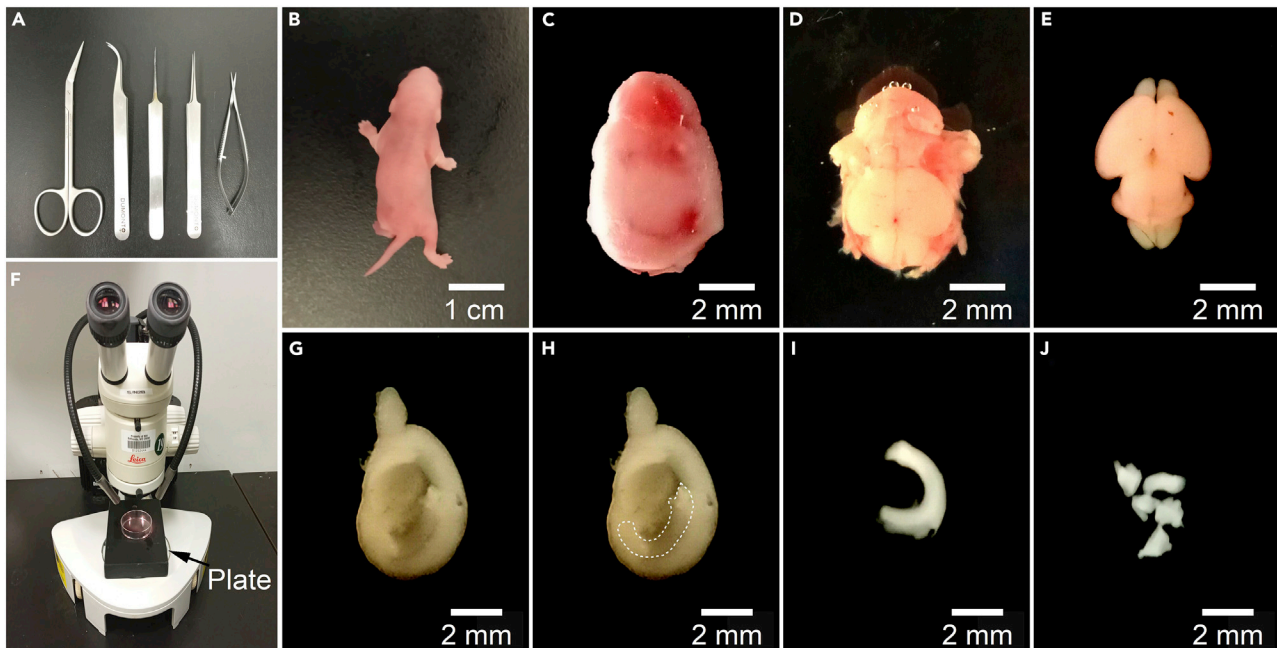13. On average, it takes 6 min to dissect 1 pup, or 30 min to dissect 5 pups.

Figure 1. Dissection of hippocampus from newborn mouse
(A) A set of surgical tools, including an angled scissors, curved tweezers, blunt tweezers, fine tweezers, and fine scissors.
(B) A P0−1 pup. Scale bar: 1 cm.
(C) An isolated head in the dish containing ice-cold Dissection Solution. Scale bar (2 mm) also applies to panels (D–J).
(D) A head after exposing brain.
(E) An isolated brain.
(F) A dissection microscope. The arrow shows a frozen-cold iron plate under a dish.
(G) The location of the hippocampus in the ventromedial region of cerebral hemisphere.
(H) Hippocampus marked with a dotted line, a "C"-shaped structure.
(I) Isolated hippocampal tissue.
(J) Chopped hippocampal tissue.

14. Use a 1-mL tip to transfer all chopped tissues into a 15-mL tube and wash them with 10 mL of ice-cold HBSS (with Ca^{2+} and Mg^{2+}) twice.
15. Remove 90% of the ice-cold HBSS before digestion.

Hippocampal digestion and dissociation

⏱ Timing: 80 min

16. Using a 1-mL tip, transfer the hippocampal tissue to a 15-mL tube containing 5 mL 37°C Papain Solution.
17. Remove any bubbles that cause the tissue to float. Incubate the tissue in the 37°C incubator for 45 min, occasionally shaking gently.
18. During this period, make up the 1/1 Solution and 10/10 Solution.
19. Using a flame-sterilized tweezers, place a 25-mm coverslip coated with mouse laminin and poly-D-lysine in each well of a 6-well plate. Keep the plate in the 37°C incubator until cell plating.
20. 45 min later, transfer the hippocampal tissue to a 15-mL tube containing 2 mL of 1/1 Solution to stop digestion.
21. Use a 1-mL tip to triturate the digested tissue 2–3 times, allowing the tissue to settle to the bottom of the tube before each trituration (Figure 2). Then, gently pipette them up and down 5 times until all pieces are broken up uniformly. After trituration, the suspension should appear cloudy without any large pieces of tissue (Figure 2D).
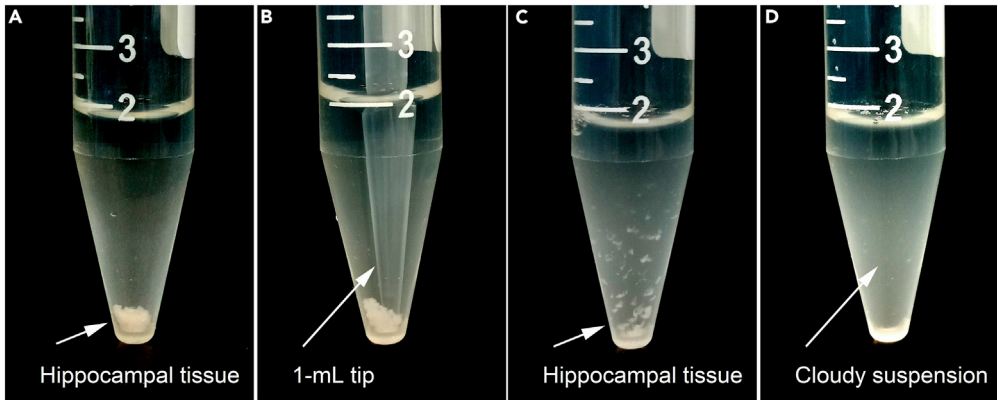
Figure 2. Trituration of hippocampal tissue
(A) Before trituration, the digested hippocampal tissue in 2 mL of 1/1 Solution settled to the bottom of the tube.
(B) Using a 1-mL tip to triturate the tissue.
(C) After one trituration, hippocampal tissue becomes smaller pieces.
(D) After trituration two more times, the suspension appears cloudy without any large pieces left.

> ⚠ CRITICAL: Trituration is critical to cell yield. Improper trituration will cause low yield or cell lysis. An effective trituration is detailed as such: set the desired volume of a 1-mL pipette gun to 750 μL; depress the plunger to the first stop; immerse the tip in the solution, firmly push the tip against the bottom of the tube, then tilt the tip at a small angle to form a narrow gap between the bottom of the tube and the tip edge. When the depressed plunger is released slowly, the tissues are aspirated into the tip through the tight gap. Friction between the tissue and the gap dissociates the clumps of neurons into individual neurons. Move the tip away from the bottom of the tube and gently depress the plunger to push the suspension out of the tip. Repeat the trituration 2–3 times until the large pieces of tissue disappear.

22. At RT (22°C–24°C), spin down the suspension at 100 × g for 5 min. Carefully aspirate off 95% of the supernatant.
23. Resuspend the pellet with 2 mL of 1/1 Solution and pipette up and down 5 times with a 1-mL tip until the pellet is completely dissociated.
24. Allow the suspension to settle for 10 min at RT in the tissue culture hood. Undissociated tissue will sink at the bottom of the tube.
25. Gently remove 95% of the supernatant and layer it on the surface of 10 mL of 10/10 Solution in a 15-mL tube, rotating the tube while layering to achieve an even distribution.
26. At RT, spin down at 100 × g for 10 min. To count cells, remove 98% of the supernatant and resuspend the pellet with 3 mL of Plating Medium (3 is the dilution factor, see step 28). Pipette it up and down 5 times.

Cell plating

⏱ Timing: 20 min

27. Add 10 μL of the cell suspension to the hemocytometer to count the cells under a light microscope (10×).
28. Count the cells in a square chamber (1×10^{-4} mL = 1 mm in length × 1 mm in width × 0.1 mm in depth) (black dash box, Figure 3A). For accuracy, count the cells in each of four square chambers, use the mean value to estimate the total yield with the following formula:

$$\text{Total yield(cells)} = \frac{\text{Cells per square chamber}}{1 \times 10^{-4}(\text{volume of a square chamber, mL})} \times 3(\text{dilution factor, mL})$$

Note: A typical yield is $4.0–6.0 \times 10^5$ hippocampal cells per pup.

29. Use the Plating Medium to make up a 3.0×10^5 cells/mL (4.0×10^5 cells/mL for EM) suspension.

   Note: 1) Cells in the suspension include neurons and glial cells. It is difficult to distinguish between them under a microscope. Cells with short neurites are neurons (Figure 3F). 2) 10%–20% of cells in the suspension are dead, which will be excluded from total.

30. Add 2 mL of the cell suspension to each well of a 6-well plate (1 mL/well for a 12-well plate or 0.5 mL/well for a 24-well plate). Make sure the entire coverslip is covered by the suspension.
31. Keep the plate in the 5% CO_2, 37°C incubator (Figure 3B).

Maintenance of cultured cells

⏱ Timing: 20 min

32. Cells will be cultured in the incubator from the day of plating to day 21 *in Vitro* (DIV21). In this period, check the cells under the light microscope (10×) every 2–3 days; feed them every 3–4 days by replacing 50% of the medium in wells with pre-warmed Feeding Medium each time.
33. Timeline of maintaining the cultured cells.
    a. At DIV0, plate the cells.
    b. At DIV2–3, to limit proliferation of glial cells, add Cytosine β-D-arabinofuranoside (Ara-C), a post-mitotic inhibitor, to the medium. Its final concentration is 2–3 μM.

   ⚠ CRITICAL: 1) Glial cells support the development and growth of neurons in culture. Overgrown glial cells, however, inhibit neuronal growth and cause them to intermingle with neurons, making imaging experiments more difficult. Therefore, application of Ara-C is one of the critical factors in good culture. 2) Prepare 2 mM Ara-C solution, store in the −20°C freezer, best used within 1 year.

    c. At DIV7, transfect the cells (see steps 34–41).
    d. At DIV14, neurons are mature and ready for imaging experiments. Most coverslips will be used within one week (DIV14−DIV21).
    e. After DIV21, the remaining coverslips will be discarded due to the gradual decline in health and SypH expression of neurons.

Transfection

⏱ Timing: 1 h 15 min

34. Use Lipofectamine as a carrier to transfect synaptophysin-pHluorin (SypH) into the hippocampal neurons at DIV7 (Figure 4). Cytomegalovirus (CMV) as a promoter is inserted into a pcDNA3 vector.

   Note: SypH, a pH-sensitive fluorescent protein, disperses on the interior of the vesicle membrane (Figure 6A). pH values in the vesicle lumen and extracellular environment are 5.5 and 7.2, respectively. Vesicle exocytosis induces de-acidification of the vesicle lumen, wherein pH increases from 5.5 to 7.2, which activates the SypH to produce the fluorescence. After vesicle endocytosis, the vesicular lumen is re-acidified as H^+ is pumped into the vesicle lumen, and pH decreases from
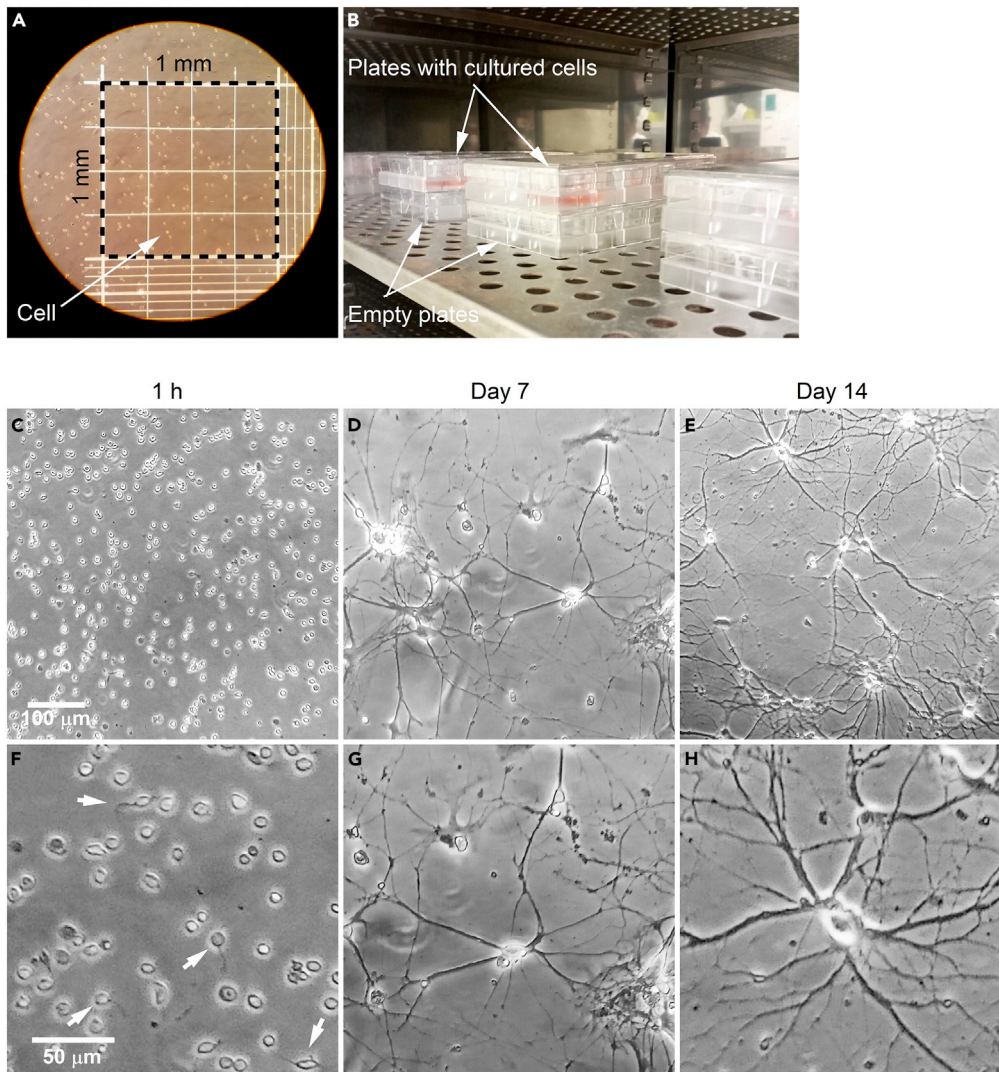
Figure 3. Culture and development of hippocampal neurons
(A) Under a light microscope (10×), the cells within a square chamber (marked with a black dash box) of the hemocytometer will be counted before cell plating. Bright dots are cells.
(B) In a 37°C, 5% CO_2 incubator, 6-well plates with coverslip-plated neurons are placed on the top of empty 6-well plates.
(C) Under a light microscope (10×), cultured neurons were imaged at 1 h after cell plating. Scale bar (100 μm) also applies to panels (D and E).
(D) Cultured neurons at DIV7 are ready for transfection.
(E) Cultured neurons at DIV14 are mature and ready for imaging experiments.
(F–H) Zoomed-in views of neurons shown in (C, D, and E). Arrows in (F) show neurons with neurites. Scale bar (50 μm) applies to panels (F, G, and H).

7.2 to 5.5, which quenches the SypH. Thus, changes in the fluorescence of SypH reflect exocytosis and endocytosis at high temporal resolution (Zhu, et al., 2009).

35. Make Medium A. For each well, mix 150 μL of Opti-MEM medium, 4 μL of Lipofectamine PLUS reagent, and 2 μg of SypH plasmid DNA in a 15-mL tube (For double transfection, such as SypH and Cre-mCherry, add 1.5 μg for each plasmid).

36. Make Medium B. For each well, mix 150 μL of Opti-MEM medium and 4 μL of Lipofectamine LTX reagent in a 15-mL tube.

DNA prepariation	Lipofectamine transfection
Component A	1.5-2 µg plasmid + 4 µl Plus + 150 µl Opti-MEM
Component B	4 µl Lipofectamine LTX + 150 µl Opti-MEM
Mix	5 min before mix, and 15-20 min after mix, RT

Cell treatments	Lipofectamine transfection
Starvation	N/A
Treatment	300 µl mixed solution to each well
Incubation	40 min, 37 °C
Stop reaction	Change medium
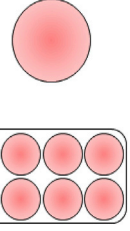
Figure 4. Lipofectamine transfection procedures

37. Incubate Medium A and B at RT for 5 min; mix them to make plasmid medium. Incubate the plasmid medium at RT for 15–20 min in the tissue culture hood.
38. Transfer 1 mL/well medium from the plate containing coverslip-plated neurons (Plate #1) to the corresponding wells of a new plate (Plate #2).
39. Add 1 mL/well fresh Feeding Medium to Plate #1 and Plate #2, respectively. Each well of Plate #1 and Plate #2 will contain 2 mL medium (1 mL old medium and 1 mL fresh medium).
40. Add 300 µL/well plasmid medium to Plate #1. Incubate the plate in the 37°C incubator for 40 min.
41. Using flame-sterilized forceps, gently transfer the coverslips from Plate #1 to the corresponding wells of Plate #2. Incubate Plate #2 in the 37°C, 5% CO_2 incubator (Figure 3B).

Note: The expression level of SypH in neurons can be checked under the fluorescent microscope.

42. At DIV13-15, neurons are mature and ready for imaging experiments. Typically, there are 5–20 SypH (+) neurons on a 25-mm coverslip, 2–5 SypH (+) neurons within imaging chamber.

Note: At DIV0, about 6×10^5 cells were added to each well of a 6-well plate (Figures 3C and 3G). During two weeks of culture, the total number of cells attached on coverslip decreases significantly, due to natural death, cell losing attachment, medium changing, and transfection. At DIV14, only 200–500 living neurons attach on a 25-mm coverslip (Figures 3E and 3H).

Part II. Acquisition and analysis of epifluorescence imaging
Study endocytosis at live individual boutons by monitoring changes in fluorescent signal under a microscope and recording with a camera. In our study, we used the Nikon Eclipse Ti-E microscope and the Andor camera (iXon X3 897 EMCCD).

Setup and setting

© Timing: 20 min

43. Choose microscope settings.
    a. Set the filters for detecting the SypH signal (excitation maximum at 475 nm; emission maximum at 509 nm).
    b. Select an oil objective (60 ×, 1.4 NA). Add a drop of immersion oil (Nikon, Type F).
44. Configure the software (NIS-Elements AR 5.20.02).
    a. Set the exposure time as 100 ms, exposure frequency as 10 Hz.
    b. Set the gain of A/D converter as 1, pixel readout rate as 10 MHz, pixel depth as 14-bit, no pixels binning for capture, and pixels binning 2 × 2 for "Live" window.
    c. Set time-lapse imaging: 3 min recording with continuous capture, which will cover fluorescence activities in the resting condition before stimulation (0–30 s), during the stimulation (30–40 s), and in the following recovery after stimulation (40–180 s) (Figure 6C).
    d. Simultaneously export a trigger signal to the stimulator when the acquisition starts.
45. Choose stimulation settings. Exocytosis and endocytosis can be induced by field stimulation. For example, a 20-mA train stimulation (200 1-ms pulse at 20 Hz) is generated from a stimulator (Model 2100, A-M Systems) and delivered to platinum electrodes in the imaging chamber through the stimulus isolation unit (Model SIU-102, Warner).

    Note: 1) Explanation for 20 mA train stimulation (200 1-ms pulse at 20 Hz), 20 mA: current strength of pulses; 200: the number of pulses in a train stimulation; 1-ms pulse: each pulse with 1 ms duration; 20 Hz: 20 pulses per second or 50 ms interval between two adjacent pulses. The train stimulation can trigger the release of vesicles, including vesicles in the readily releasable pool and replenished vesicles from the recycling pool, in boutons of hippocampal neurons. 2) The strength of pulses can be altered by adjusting the current output from the stimulus isolation unit. The number of pulses in the train stimulation, single pulse width, and frequency can be modified by adjusting the train width, the pulse duration, and the pulse interval of the stimulator, respectively. 3) Usually, there is a 30 s delay between trigger signal and train stimulation for baseline recording.

Position the coverslip on the stage of the microscope

⏱ Timing: 5 min

46. Assembly of the coverslip-chamber unit.
    a. The coverslip-chamber unit includes four parts, including a clamp, imaging chamber, coverslip, and platform (Figure 5).
    b. Smear high-vacuum silicone grease (Sigma) on the bottom of the chamber (RC-21BRFS, Warner) (Figure 5A) and the top of the platform (Model P2/PH2, Warner) (Figure 5B) with a #4 painting brush to seal the gaps between the chamber, coverslip, and platform.
    c. Using fine forceps to mount a coverslip with plated neurons on the top of the platform (Figure 5C) and cover it with the chamber (Figure 5D). Gently press on the chamber to ensure a tight seal.

    △ CRITICAL: Keep 100–200 μL of Feeding Medium on the coverslip to prevent the glass from drying.

    d. Add a clamp to the top of the chamber and tighten the 4 screws with a screwdriver to fix the chamber in place on the platform (Figure 5E).
    e. Add 250 μL of Saline Solution to the chamber (Figure 5F). Dry the bottom side of coverslip with kimwipes. During imaging experiments (30–45 min), it is unnecessary to perfuse the cells.

47. Ensure no leakage, then position, and fix the coverslip-chamber unit on the stage of the microscope. Connect the chamber to the output wires from the stimulus isolation unit for field stimulation.
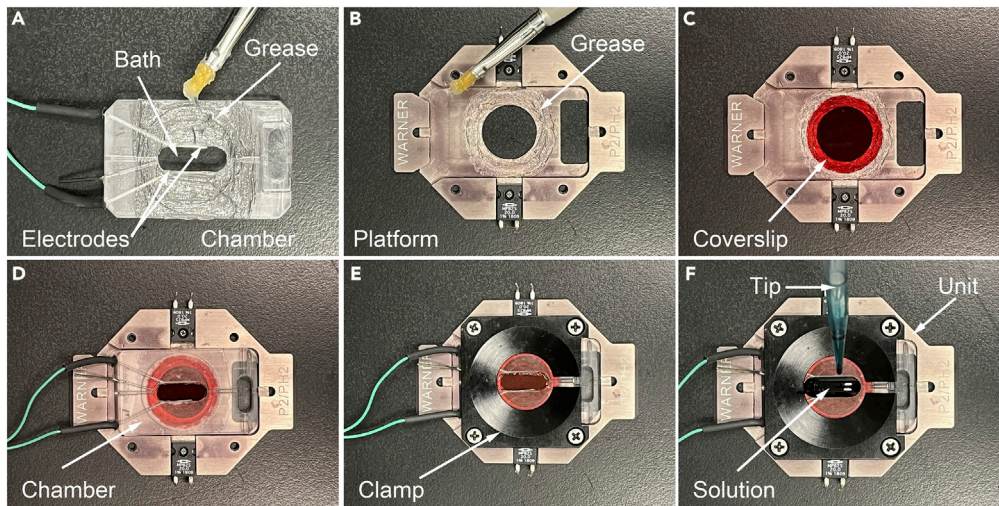
Figure 5. Preparation of the coverslip-chamber unit
(A) Bottom view of the imaging chamber. Two parallel platinum electrodes are used for field stimulation. The bath volume is 260 μL. Smear the grease on the bottom of the chamber.
(B) Smear the grease on the top of the platform.
(C) Mount a coverslip (colored with red to recognize it easily) on the top of the platform.
(D) Put the chamber on the coverslip, with the bottom of the chamber with the grease facing the coverslip. The coverslip forms the floor of the chamber.
(E) Put a clamp on the top of the chamber and tighten the screws to fix the chamber on the platform.
(F) Coverslip-chamber unit was built. Add 250 μL of Saline Solution to bath.

> △ CRITICAL: If the Saline Solution is leaking from the gap between the chamber and cover-slip, it will mix with the immersion oil under the coverslip. The mixture of leaked Saline Solution and immersion oil will result in changes in the refractive index that cause lack of focus during recording.

Collection of imaging data

⏱ Timing: 45 min

In general, data in an independent experiment are generated by the first round of stimulation on each coverslip.

48. Turn off the room lighting and keep the acquisition window in a dark scheme to reduce ambient light.
49. Detect the SypH (+) neurons.
    a. Under the simple white field, focus the sample by ocular observation.
    b. Under the epifluorescent light (Intensilight, C-HGFI; power set as 100% illumination), look for SypH (+) neurons and branches by ocular observation as soon as possible.
    c. Reduce the epifluorescence light to 12.5% illumination, switch light path to camera, observe SypH (+) neurons and branches on active "Live" window.

> △ CRITICAL: In resting condition, if the fluorescent signals in the boutons are round or oval and those of branches are dim, blurry, and continuous, the neuron is a good candidate for the experiment; if they are bright, sharp, and discontinuous, the neuron may be sick or already dead, and you should not use it. Change to another coverslip if SypH (+) neurons cannot be found.
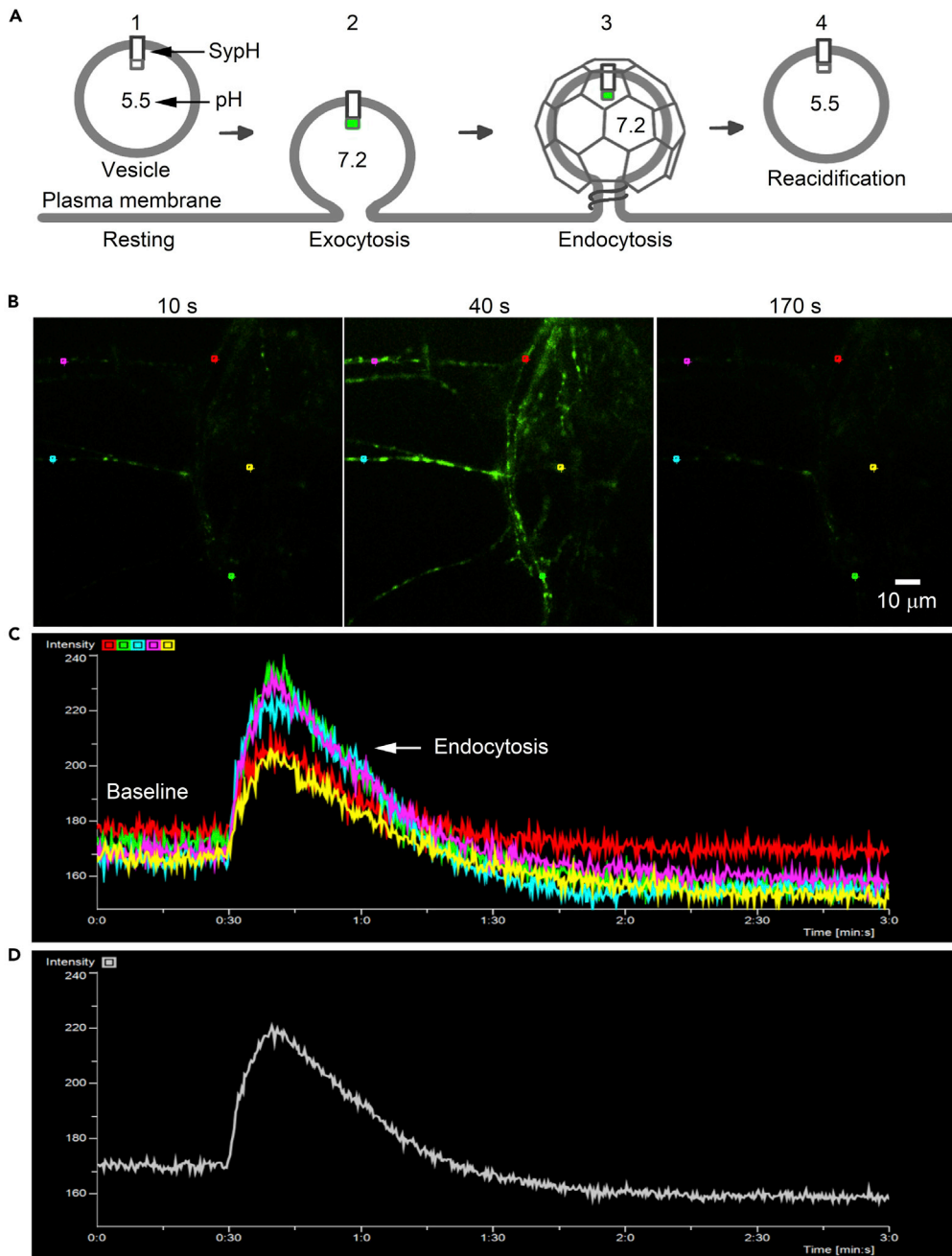
Figure 6. Fluorescence images

(A) Diagram of SypH tracking exocytosis and endocytosis. 1: In resting condition, SypH is quenched by low pH ($pH_{vesicle}$ = 5.5) in the vesicle (see Figure 6B, left). 2: When the vesicle fuses with the plasma membrane, $pH_{vesicle}$ increases to 7.2 because the protons move out of the vesicle, which activates SypH to produce the fluorescence (see Figure 6B, center). 3: Vesicle endocytosis. 4: After endocytosis, vesicles are re-acidified, which quenches SypH again (see Figure 6B, right). Therefore, exocytosis and endocytosis can be tracked in real-time by monitoring changes in SypH signals (see Figure 6C).

(B) Snapshots at 10 s (left), 40 s (center) and 170 s (right) in a 180-s imaging video. ROI squares (2 μm × 2 μm) marked with different colors represent five boutons, which were visualized by dim fluorescence puncta. The intensity of the fluorescence signal reached its maximum at 40 s (center) at the end of a 10-s train stimulation (200 1-ms pulse at 20 Hz). Scale bar (10 μm) applies to all photos in panel B.

(C) In Time Measurement window, fluorescence intensity traces were collected from 5 corresponding ROIs in (B). Their baselines, from 0 to 30 s, have different intensity levels. During a train stimulation (200 1-ms pulse at 20 Hz), from 30 s to

Figure 6. Continued

40 s, the intensity of signals increases, representing the net outcome of exocytosis and endocytosis. After 40 s, signals decay slowly, reflecting endocytosis.

(D) The mean trace is averaged from the 5 traces shown in panel (C).

50. Switch off epifluorescence light temporarily and mark 20–50 expected boutons, visualized by dim fluorescence puncta, with 2 μm × 2 μm square markers on inactive "Live" window. Marking takes several minutes. Boutons will be identified by the increase of fluorescence intensity induced by stimulation.

    ⚠ CRITICAL: Dimming of fluorescent signals may be caused by 1) bleaching induced by intensive stimulation and long exposure to the epifluorescence light; 2) unhealthy cells collected from the knock-out mice. To avoid this issue, data generated only by the first round of stimulation will be considered for analysis. After that, the corresponding coverslip with cultured neurons will be discarded.

51. Data acquisition.
    a. Switch on the epifluorescent light, double-check the focus by observing the active "Live" window and start an acquisition.
    b. Based on changes in the fluorescent signals shown on the window (Figure 6C), identify which square markers represent the boutons and observe if endocytosis rate is affected or not.

    ⚠ CRITICAL: If boutons have not responses to stimulation, check the system to make sure the stimulation is indeed delivered from the stimulator to the imaging chamber. Otherwise, try another coverslip.

    c. Save an acquisition.

Quantitative analyses of epifluorescent data

⏱ Timing: 1–2 h per experiment

In our studies, the Nikon analysis software (NIS-Elements AR Analysis) was used for offline analysis. Free software, such as ImageJ, can also be used in analysis.

52. Identify and mark the boutons with square markers (2 μm × 2 μm region of interest (ROI)) (Figure 6B). Ensure each ROI large enough to cover the largest changes in fluorescence induced by vesicle fusion and fission, which will rule out the artifacts induced by movement of SypH along the plasma membrane.

53. Because the boutons of a neuron have different responses to the same stimulation, more than 20 boutons in one field of view will be randomly selected for data analysis per experiment.

    ⚠ CRITICAL: 1) In most cases, one field of view usually contains enough boutons (>20) for analysis. If not, simply exclude those recordings. For unhealthy cells from the KO mice, it is less probable that 20 boutons will be visible in one field of view. This problem will be solved by increasing the density of cells during plating or try more cultures. It is not recommended to collect data generated by multiple rounds of stimulation in different fields of view in an experiment. 2) The stimulation, exposure time, and intensity of epifluorescent light used in our previous studies did not cause significant bleaching. Similar fluorescent responses can be induced by 3–5 rounds of train stimulation at WT neurons. If bleaching happens, affected boutons are not considered in analysis. 3) Low expression of fluorescence will make boutons have small responses to stimulation. Increasing the amount of

plasmid or using different carriers in transfection will improve the expression of SypH in cultured neurons.

54. To estimate background fluorescence in the region without cells, select an area around a marked bouton and mark it with the square maker (2 μm × 2 μm). Because boutons are visualized by puncta with different intensities of fluorescence, about 20 background areas are selected per experiment, each marked bouton having its own marked background.
55. Export the averaged data of bouton and background signals (Figure 6D) to Excel.
56. To compare data from different experiments, the fluorescence change (ΔF) of boutons is normalized in Excel using the following formula:

$$\text{Normalized } \Delta F\,(\%) \;=\; \frac{F_{max} \;-\; F_{baseline}}{F_{baseline} \;-\; F_{background}} \times 100$$

Note: F_{max} and $F_{baseline}$ refer to maximal fluorescent increase after stimulation and baseline fluorescence before stimulation, respectively, and $F_{background}$ refers to background fluorescence.

57. In each experimental condition, about 10 independent experiments from 3 cultures should be conducted to minimize the effects of random variation on the data.
58. Use the endocytosis initial rate ($Rate_{decay}$), the amount of fluorescence decay during the first 4–10 s after stimulation (Figure 7A), to measure the rate of endocytosis quantitatively in hippocampal synapses in different experimental conditions.

⚠ CRITICAL: In control condition, endocytosis decay can be fit with a single exponential function and measured quantitatively with a time constant (τ). When endocytosis is inhibited, fluorescence decay slows down; the fluorescent signals usually do not return to baseline in each time window. Therefore, τ will not be used in this study of endocytosis because the value of τ is difficult to determine when endocytosis is inhibited.

59. In Igor software, $Rate_{decay}$ is calculated by the following formula:

$$Rate_{decay}\,(\%\,/\,s) \;=\; \frac{\Delta F\,(\%)}{4\text{ s}\;-\;10\text{ s after stimulation}}$$

Note: ΔF refers to the change in fluorescence density within 4–10 s after stimulation (Figure 7A). A smaller $Rate_{decay}$ represents slower endocytosis.

60. Determine if mutation affects endocytosis by comparing the value of $Rate_{decay}$ of the control group with that of the mutation group. Figures 7B–7D shows a real case.

Part III. EM acquisition and analysis
Endocytosis is studied by observing the formation of individual vesicles and endosomes from the plasma membrane under an electron microscope. A typical experimental design includes four pre-conditions before a fixative is added (Figure 8A): 1) resting condition without 90 mM K^+ stimulation; 2) no recovery condition with 90 mM K^+ stimulation for 1.5 min only; 3) short recovery condition with 90 mM K^+ stimulation for 1.5 min and 3 min recovery after KCl washout; 4) long recovery condition with 90 mM K^+ stimulation for 1.5 min and 10 min recovery after KCl washout. Horseradish peroxidase (HRP) is applied for 1.5 min during the application of 90 mM K^+ solution to label endocytosed vesicles. In resting condition, HRP is also added for 1.5 min before fixation.

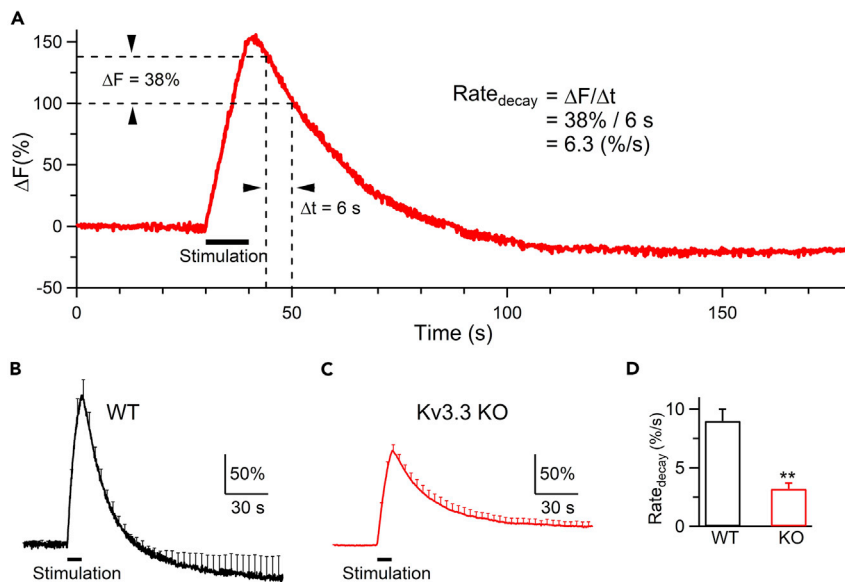*Cell culture and transfection*

⊙ Timing: 5–6 h

Figure 7. Analysis of endocytosis rate

(A) In Igor software, a sampled trace shows how the initial rate of endocytosis (Rate$_{decay}$) was calculated. Exocytosis and endocytosis were induced by a train stimulation (black bar, 200 1-ms pulse at 20 Hz). The same stimulation also applies to (B and C).

(B and C) Fluorescence response (mean + SEM) induced by stimulation in WT (B, n=17) and Kv3.3 KO (C, n=15) boutons at 34–37°C. Kv3.3: a voltage-dependent potassium channel.

(D) Summary of Rate$_{decay}$ (mean + SEM) collected from B and C. **: $p < 0.01$; t test (compared to WT). Note: Data in Figure B–D indicate that Kv3.3 enhances the vesicle endocytosis in hippocampal synapses. Data in Figure B–D have been modified from Wu et al. (2021).

61. Cell culture and transfection.
    a. Preparation of coated plates for cell plating in EM experiments.
       i. Coat the plate 3 h before dissection.
       ii. Add 300 μL of filtered poly-L-lysine solution to each well of the 24-well plate.
       iii. Incubate the plate at 37°C for 2 h.
       iv. Aspirate the poly-L-lysine solution.
       v. Wash the plate 3 times with sterilized water for 10 min each.
       vi. Air dry for 30 min in the tissue culture hood, store in the 37°C incubator, and start the dissection.
    b. Hippocampal harvest, digestion and dissociation is the same as the protocol described in steps 1–29.
    c. Add 0.5 mL of the cell suspension (4.0×10^5 cells/mL) to each well of a 24-well plate and store in the 5% CO_2, 37°C incubator.
    d. For wild-type and conventional knockout mice, the cultured neurons do not need any transfection at DIV7. At DIV14, neurons are mature and ready for EM experiments.

⚠ CRITICAL: For conditional knockout mice, it is necessary to transfect the Cre gene into hippocampal neurons at DIV7 to remove LoxP-flanked gene(s). However, the transfection rate is only ∼5% when Lipofectamine is used as a carrier for transfection of the Cre gene. To increase transfection efficiency, adeno-associated virus (AAV) can be used as a plasmid carrier to transfect the Cre gene into cells at DIV7. The transfection efficiency will be more than 95% at DIV14, which is extremely helpful for EM experiments.
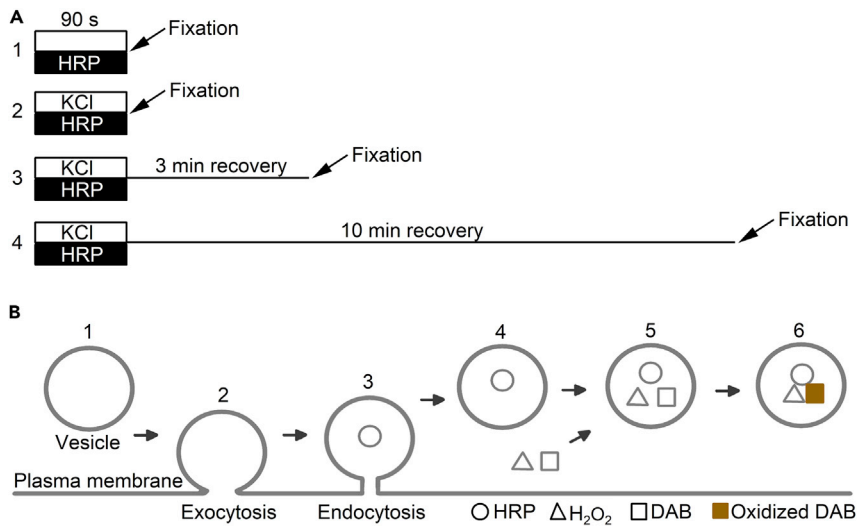
Figure 8. Four experimental conditions before fixation in the EM protocol

(A) HRP staining with or without application of high K^+ solution to the samples before fixation.

(B) Diagram of HRP staining a vesicle. 1: Vesicle in resting condition. 2: Vesicle exocytosis. 3: HRP enters the vesicle during endocytosis. 4: HRP in endocytosed vesicle. DAB and H_2O_2 penetrate the plasma membrane into the cytosol. 5: DAB and H_2O_2 enter the vesicle. 6: Vesicle stained by the oxidized DAB.

Stimulation, staining, and primary fixation

⏱ Timing: 2 days

62. Prepare HRP (5 mg/mL) Saline Solution and HRP (5 mg/mL) 90 mM KCl Solution.

> *Note:* HRP is an enzyme that catalyzes the oxidation of various substrates, such as 3,3'-diaminobenzidine (DAB), using hydrogen peroxide (H2O2). Oxidized DAB is an insoluble product with a brown color. DAB and H2O2 can enter the cytosol by penetrating the plasma membrane, but HRP cannot. HRP enters the cytosol only by vesicle endocytosis. Therefore, oxidation of DAB happens only in HRP (+) vesicles. Thus, HRP can be used to track endocytosed vesicles in imaging experiments (Figure 8B).

63. Dilute glutaraldehyde from 70% to 4% and 2% by adding 0.1 M sodium cacodylate buffer (pH 7.4).

> ⚠ CRITICAL: 70% glutaraldehyde is toxic and sticky. Wear a lab coat, gloves, and safety goggles; stir the glutaraldehyde solution in a fume hood while diluting.

64. At DIV14, transfer all culture medium in the 24-well plate containing the plated neurons (Plate #1) to the corresponding wells of a new 24-well plate (Plate #2).

> ⚠ CRITICAL: Care must be taken to avoid drying of the neurons.

65. Experiment for the resting condition (condition 1) (Figure 8A).
    a. Add 300 μL of the HRP Saline Solution to each well of Plate #1 for 1.5 min at RT. The HRP (+) vesicles in this condition will be rare because the frequency of spontaneous endocytosis is extremely low.
    b. Aspirate the HRP Saline Solution and wash the cells with 500 μL of the PBS.
    c. Aspirate the PBS and add 500 μL of 4% glutaraldehyde to fix the cells.

⚠ CRITICAL: For a 24-well plate, we divide the wells with plated cells into 4 groups, which correspond to the four preconditions in EM experiments. Each group includes 3–4 wells.

66. Experiments for other three high KCl stimulation conditions.
    a. Add 300 μL of the HRP 90 mM KCl Solution to each well of Plate #1 for 1.5 min at RT, to depolarize all neurons in the wells. Uptake of HRP into the vesicles will catalyze oxidation of DAB by H_2O_2 in endocytosed vesicles as explained in step 62.
    b. Aspirate the HRP 90 mM KCl Solution and wash the cells with 500 μL of the PBS once to stop depolarization of the neurons completely.
    c. After aspirating the PBS,
        i. Condition 2: Add 500 μL of 4% glutaraldehyde to fix cells immediately after the PBS is removed. There is a 5–10 s interval between stimulation and fixation.
        ii. Condition 3 and 4: Transfer the culture medium back from the wells of Plate #2 to the corresponding wells of Plate #1 for a 3 min recovery (condition 3) or 10 min recovery (condition 4); replace the culture medium in the wells of Plate #1 with 500 μL of 4% glutaraldehyde to fix the cells.
67. All fixations last 2 h at RT in the fume hood.

Note: Glutaraldehyde is used to fix the samples, so that structures can be preserved for EM observation. Fixation kills the cells. EM images are snapshots of living samples before fixation.

68. Wash with 500 μL of 0.1 M sodium cacodylate buffer 3 times and store the plate at 4°C overnight (12 h).
69. Allow the plate to equilibrate to RT.
70. Stain the fixed cells with 500 μL of the DAB Solution at 37°C for 30 min (Figure 8B).

Note: DAB will be oxidized in HRP (+) vesicles. Oxidized DAB forms brown precipitate, which can be detected by EM.

71. Wash the fixed cells 3 times with 500 μL of 0.1 M sodium cacodylate buffer for 7 min each.
72. Aspirate the sodium cacodylate buffer and add 500 μL of 2% glutaraldehyde.
73. Temporarily store at 4°C before post-fixation.

EM sample processing and thin section preparation

⏱ Timing: 5 days

Preparation of thin sections containing boutons is critical in the EM experiment. Given the difficulty of this step, experience will help immensely to accelerate it. In the present study, this step was performed by researchers in the dedicated EM facility at the National Institute of Neurological Disorders and Stroke (NINDS).

74. Remove 2% glutaraldehyde, wash with 500 μL of 0.1 M sodium cacodylate buffer 3 times for 7 min each.
75. Incubate the fixed cells in 1% OsO_4 (in 0.1 M sodium cacodylate buffer) on ice for 1 h, covered with foil. OsO_4 will contrast the image.

⚠ CRITICAL: OsO_4 is acutely toxic. Wear a lab coat, protective goggles, and gloves; keep the sample in the chemical fume hood.

76. Wash the samples three times with 500 μL of 0.1 M sodium cacodylate buffer on ice for 7 min each.

77. Uranyl acetate staining.
    a. Wash the samples 3 times with 500 μL of 0.1 N sodium acetate buffer (pH 5.0) for 7 min each.
    b. Incubate the samples with 500 μL of 0.25% uranyl acetate (UA, in 0.1 N sodium acetate buffer) for 1 h at 4°C. The samples are covered with foil, which keeps them in the dark to avoid UA precipitation.
    c. Wash the samples 3 times with 500 μL of 0.1 N sodium acetate buffer for 7 min each. Uranyl acetate staining will increase image contrast.
78. Dehydration.
    a. Dehydrate the samples with 500 μL of 50%, 70%, and 90% ethanol in a fume hood for 7 min each.
    b. Wash the samples 3 times with 500 μL of 100% ethanol for 7 min each.

   △ CRITICAL: Replace the water content in the samples with ethanol to allow resin infiltration, as resins are hydrophobic.

79. Resin embedding.
    a. Incubate the samples with 500 μL of 1:1 epoxy resin/(100%) ethanol on a rocker plate for 30 min at RT.

   Note: 1) Epoxy resin penetrates the cell and occupies all the space in the cell. 2) Some labs prefer to use acetone as a transition solvent instead of ethanol.

    b. Incubate the samples with 500 μL of 2:1 epoxy resin/ethanol on a rocker plate for 30 min at RT.
    c. Incubate the samples with 500 μL epoxy resin in a 50°C oven for 10 min.
    d. Exchange epoxy mixture with fresh 100% epoxy resin and incubate at 50°C for 1 h. Repeat it one more time.
80. Polymerization. Add 500 μL of fresh epoxy resin and allow to harden in a 50°C oven overnight (12 h) and then in a 60°C oven for another 48 h.

   Note: After polymerization, the sample is hard enough to bear the pressure of sectioning.

81. Carefully select a region of interest (ROI), an area with plenty of neurites, under an inverted light microscope (Figures 9A–9C).
82. Saw out the selected ROI and surrounding area (about 4 mm × 8 mm) with a jeweler's saw. Remove sawed-out sample from plastic bottom of plate using a pair of pliers.
83. Place the sample on a chuck holder in an ultramicrotome (Leica Ultracut S), where it is manually trimmed to a trapezoid shape, with less than ~0.5 mm wide and ~1 mm long on the face side; the sample is sectioned extremely thinly (~70 nm thick) using a diamond knife (its edge is parallel to the surface of the block) (Figure 9D). The sections are then collected on individual grids (400 mesh copper grid) and air dried overnight (12 h).

   △ CRITICAL: Total of 5 copper grids are collected every ~5 sections, such as the 1st, 6th, 11th, 16th and 21st sections, to encompass neurite depth at different levels. Other sections are discarded.

84. Stain with 3% lead citrate for 5 min to enhance the contrast of the sections.
85. Wash the grids with ddH$_2$O for 10–12 s, wick dry them with filter paper, then air dry. The stained sections on the grids are now ready for examination under the electron microscope.

The whole workflow (steps 62–85) for preparing the EM sample is shown in Figure 10.
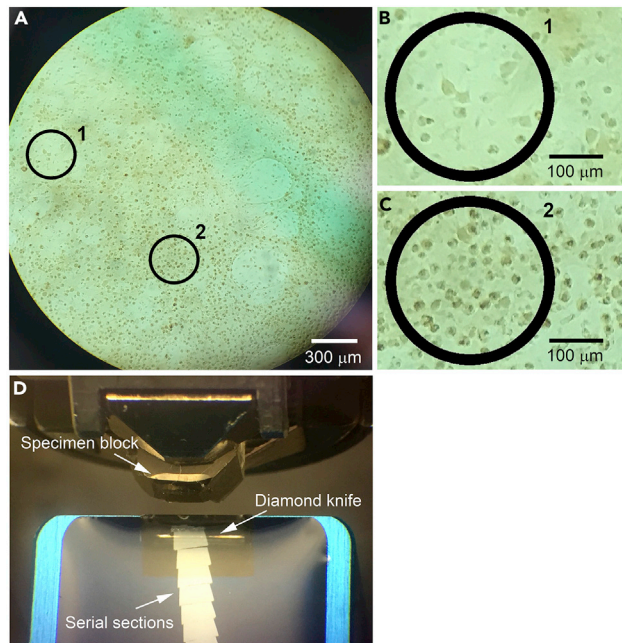
Figure 9. Determination of the regions of interest (ROIs) and preparation of the thin sections
(A) The cells (grey spots) embedded in the resin are viewed under an inverted light microscope (10×). Cells in Circle 1 and Circle 2 show a difference in their densities. Two circles have the same size (diameter = 300 μm). Scale bar: 300 μm.
(B) Zoomed-in view of Circle 1, showing low density of neuronal somas and with many interconnecting neurites. The areas like Circle 1 will be the ROIs for further thin section cutting. Scale bar: 100 μm.
(C) Zoomed-in view of Circle 2, showing high density of neuronal somas. This kind of area may yield fewer synapses. Scale bar: 100 μm.
(D) Serial thin sections were cut by the ultramicrotome (Leica Ultracut S).

EM image acquisition

⏱ Timing: 5 h

In this protocol, examine the images with a transmission electron microscope (JEOL JEM-200CX, 120 kV) and record the images with a CCD digital camera (AMT XR-100) at a magnification of 10,000–38,000×.

86. Set the EM magnification at:
    a. 100–1000× to find the sections on the grid.
    b. 1000–5000× to choose the cells.
    c. 10000–38,000× to image the boutons.

    ⚠ CRITICAL: Two landmarks are helpful for identifying the synaptic boutons, 1) the electron dense thickening of the postsynaptic plasma membrane (the post synaptic density (PSD)), and 2) uniform sized synaptic vesicles in the presynaptic terminal opposed to the PSD.

87. Set exposure time as 60 ms, pixel binning as 4 × 4, gain of the A/D converter as 2, image average value as 2.
88. Collect the digital images of the synaptic boutons from different sections. It is better to have a clear presynaptic and postsynaptic structure in the photo. Vesicles in the presynaptic part have a round shape and clear membrane structure (Figures 11 and 12).
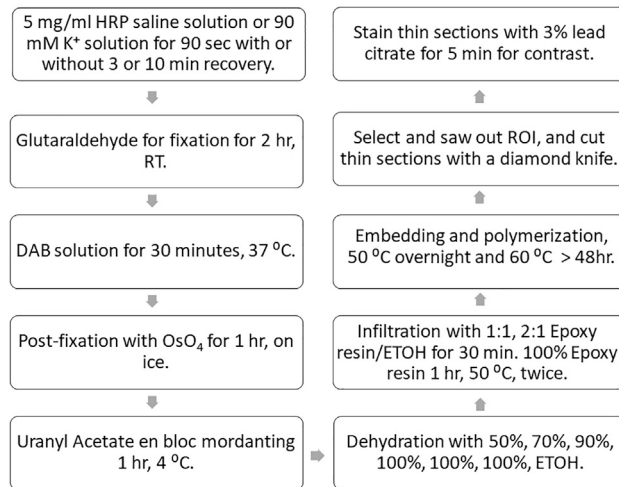
```
┌─────────────────────────────┐        ┌─────────────────────────────┐
│ 5 mg/ml HRP saline solution │        │ Stain thin sections with 3% │
│ or 90 mM K⁺ solution for 90 │        │ lead citrate for 5 min for  │
│ sec with or without 3 or 10 │        │ contrast.                   │
│ min recovery.               │        │                             │
└─────────────────────────────┘        └─────────────────────────────┘
              ↓                                       ↑
┌─────────────────────────────┐        ┌─────────────────────────────┐
│ Glutaraldehyde for fixation │        │ Select and saw out ROI, and │
│ for 2 hr, RT.               │        │ cut thin sections with a    │
│                             │        │ diamond knife.              │
└─────────────────────────────┘        └─────────────────────────────┘
              ↓                                       ↑
┌─────────────────────────────┐        ┌─────────────────────────────┐
│ DAB solution for 30         │        │ Embedding and               │
│ minutes, 37 °C.             │        │ polymerization, 50 °C       │
│                             │        │ overnight and 60 °C > 48hr. │
└─────────────────────────────┘        └─────────────────────────────┘
              ↓                                       ↑
┌─────────────────────────────┐        ┌─────────────────────────────┐
│ Post-fixation with OsO₄ for │        │ Infiltration with 1:1, 2:1  │
│ 1 hr, on ice.               │        │ Epoxy resin/ETOH for 30     │
│                             │        │ min. 100% Epoxy resin 1 hr, │
│                             │        │ 50 °C, twice.               │
└─────────────────────────────┘        └─────────────────────────────┘
              ↓                                       ↑
┌─────────────────────────────┐        ┌─────────────────────────────┐
│ Uranyl Acetate en bloc      │   →    │ Dehydration with 50%, 70%,  │
│ mordanting 1 hr, 4 °C.      │        │ 90%, 100%, 100%, 100%,      │
│                             │        │ ETOH.                       │
└─────────────────────────────┘        └─────────────────────────────┘
```

Figure 10. Workflow for preparing EM sample

⚠ CRITICAL: Image 30–50 boutons in a sample from one culture; data in each experimental condition are collected from at least 3 cultures.

Quantification and statistical analysis of EM data

⏱ Timing: 5–20 min per image

Use ImageJ, a free software, to analyze the EM images (Schindelin, et al., 2012).

89. Locate the synaptic structure, which includes the presynaptic bouton, synaptic cleft, and PSD (Figure 11). Boutons contain synaptic vesicles (diameter (d) 40 nm) and endosomes (d > 80 nm).

⚠ CRITICAL: 1) Bulk endocytosis, a large piece of membrane invagination after strong stimulation, generates endosomes, which gradually disappear as they are used to regenerate synaptic vesicles. 2) In resting condition, HRP (+) vesicles and endosomes are rare. 3) After stimulation, HRP (+) synaptic vesicles and endosomes increase, which suggests that vesicle endocytosis and bulk endocytosis occurred.

90. To study endocytosis, measure the areas of boutons and HRP (+) bulk endosomes, and count the number of HRP (+) vesicles (Figure 11).

⚠ CRITICAL: 1) Bouton size varies in different ultrathin sections. Using area of bouton normalizes the data to rule out the effects of bouton size on the results. 2) Both area and number of endosomes can reflect the bulk endocytosis. Our statistics indicate that the results based on the area were similar to those based on the number. In our studies, we measured the area of endosomes as it had a smaller variance.

91. Calculate the number of HRP (+) vesicles per bouton (μm^2) and the HRP (+) bulk endosome area per bouton (μm^2) (Figure 12).

Note: Data in each group, such as the wild-type group, will be collected from 90–150 boutons in each of the four preconditions.
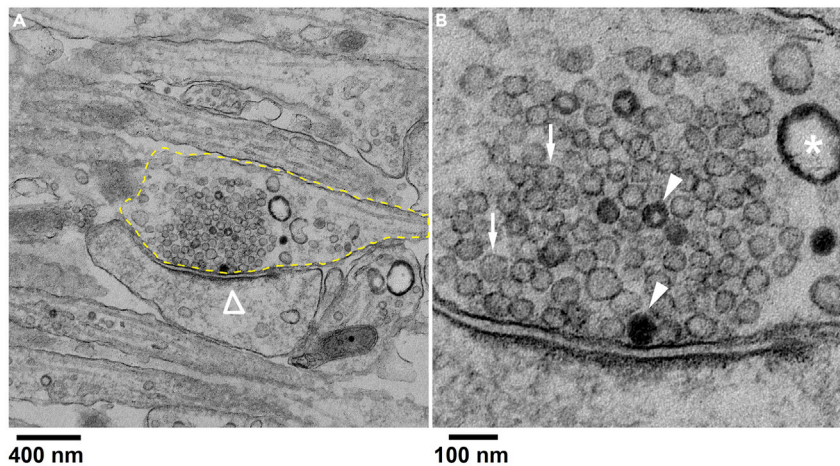
Figure 11. Ultrastructure of hippocampal neuron imaged by transmission electron microscopy
(A) The neurites of hippocampal neurons were imaged immediately after washout of 90 mM KCl and 5 mg/mL HRP (Mag: 20000×) (condition 2). A synapse is shown in the center of the picture, including the presynaptic bouton (yellow dash line), synaptic cleft, and postsynaptic compartment (empty triangle). Scale bar: 400 nm.
(B) The bouton is zoomed in for detail. Arrows: HRP (-) vesicles; arrowhead: HRP (+) vesicles (filled with the oxidized DAB); *: HRP (+) endosome. Scale bar: 100 nm.

92. Through analysis of the data, the speed of vesicle endocytosis and bulk endocytosis can be estimated. For example, in the same condition, if the number of HRP (+) vesicles per bouton (μm^2) obtained from the mutated cells after application of high K^+ is less than the value obtained from wild-type, vesicle endocytosis may be inhibited by mutants. Figure 12 shows a real case.

Note: To confirm changes in endocytosis induced by mutants, additional experiments are necessary, such as the rescue experiment.

EXPECTED OUTCOMES

Part I. Hippocampal neuron culture

Primary neuron culture has become indispensable tool in the field of neuroscience. It provides a simple network between neuron, a controllable environment, and accessibility for imaging tools to target specific parts of live neurons, making measurement of endocytosis in live conventional synapses possible. Culture quality is crucial for imaging endocytosis. When the steps are properly performed as described above, the expected culture appears as shown in Figure 3H. At DIV14, healthy individual neurons under a light microscope will have a bright soma, continuous axon, and dendrites. About 200–500 neurons can live on a 25-mm coverslip. When growth of glial cells is properly controlled, 200–500 glial cells live on a 25-mm coverslip.

Part II. Acquisition and analysis of epifluorescence imaging

When the steps are properly performed as described above, the expected result will appear as shown in Figures 6 and 7. On average, 2–5 SypH (+) neurons can be observed in the field of imaging chamber. In resting condition, more than 20 boutons can be located along the dimmer branches of the axon (Figure 6B, left). During stimulation, the intensity of fluorescence at the boutons will increase due to protons in the vesicle lumen moving to the extracellular space, reflecting the net outcome of major exocytosis and minor endocytosis (Figure 6B, center). After stimulation, the intensity of fluorescence will decrease because protons are pumped back into the vesicle lumen by the H^+ pump on the vesicle membrane, reflecting vesicle endocytosis (Figure 6B, right). Epifluorescent imaging shows the fusion and fission of vesicles with high temporal resolution in live cells. It can be used to study mechanisms that regulate the time course of exocytosis or endocytosis.
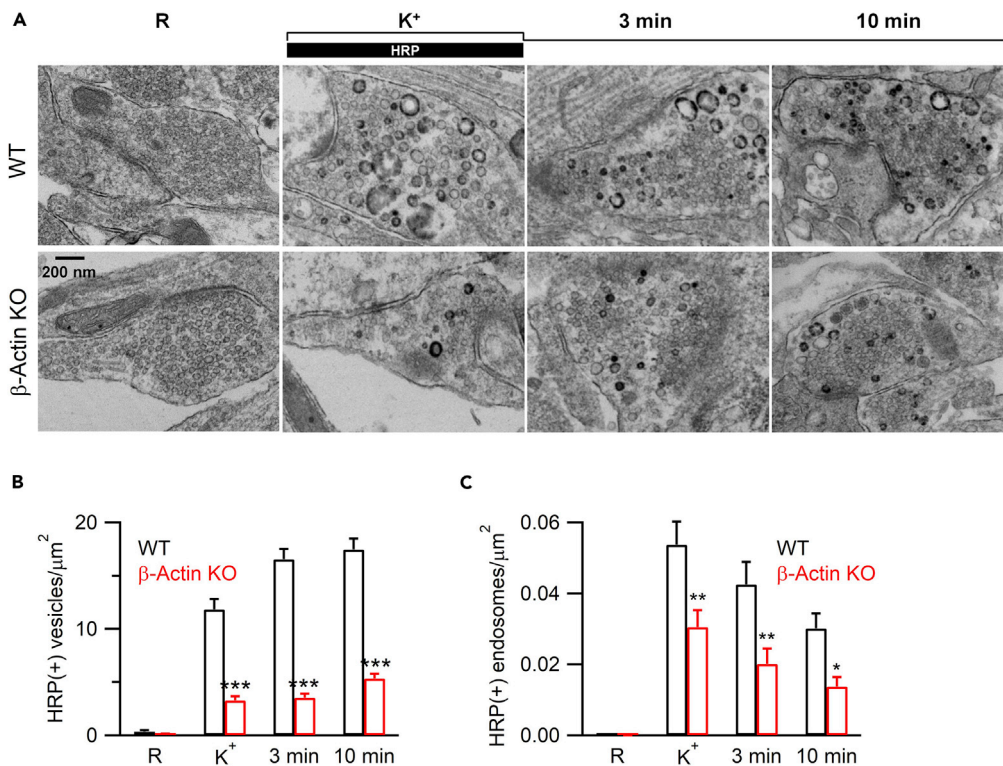
Figure 12. Ultrastructural changes in β-actin KO hippocampal boutons

(A) Electron microscopy images of wild-type and β-actin KO hippocampal boutons fixed at rest (R) and at 0 (K⁺), 3, and 10 min after the end of 1.5 min 90 mM KCl application. For R, HRP was applied for 1.5 min before fixation; for other three conditions, HRP and KCl were co-applied for 1.5 min. Scale bar (200 nm) applies to all photos in panel A.

(B and C) The number of HRP(+) vesicles (B) and bulk endosome area (C) per bouton (μm^2) is plotted versus the time before (R) and at 0 (K⁺), 3, and 10 min after the end of KCl application in control (black bars) and β-actin KO (red bars) hippocampal cultures (mean + SEM; each group was from 40–100 synaptic profiles). ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ (ANOVA). Note: These results suggest that β-actin enhanced both vesicle endocytosis and bulk endocytosis in hippocampal neurons. Data in Figure 12 have been modified from Wu et al. (2016).

Part III. EM acquisition and analysis

When the steps are properly performed as described above, the expected result should appear as shown in Figures 11 and 12. The various membrane structures in view are clear. The ultrastructure of the synapse with high density can be recognized. The typical diameter of a bouton is around 1 μm. Clear-core vesicles (40 nm) and endosome-like structures (> 80 nm) are visible in boutons. When HRP is applied, the dense aggregates (the oxidized DAB) are visible in vesicles and endosomes. Based on the number, size, percentage, and distribution of HRP (+) vesicles or endosomes in boutons in the four different conditions, the time course of endocytosis can be estimated.

QUANTIFICATION AND STATISTICAL ANALYSIS

1. The information for data processing and quantification have been detailed in steps 52–60 for fluorescent experiments and steps 88–92 for EM experiments.
2. Data are presented as means ± SEM. A *t* test with equal variance was used for analysis of fluorescent image data, and an ANOVA test for analysis of EM data.

LIMITATIONS

Epifluorescence imaging

pH values in the vesicle lumen and extracellular environment are 5.5 and 7.2, respectively. Vesicle exocytosis induces vesicle lumen de-acidification (from 5.5 to 7.2), which increases the fluorescence

intensity by activating the SypH, a pH-sensitive fluorescent protein. Vesicle endocytosis is followed by vesicular lumen re-acidification (from 7.2 to 5.5), which decreases the fluorescence intensity by quenching the SypH. Thus, changes in SypH fluorescence directly reflect the variation of pH values in vesicles only. There is a delay between a vesicle moving in or out of a synapse and changes in its acidity. Potentially, changes in SypH signals may not be caused by vesicle fusion and fission, but rather by other means of changing pH. Also, the low resolution of epifluorescence imaging limits our ability to visualize endocytosis dynamics at the single vesicle level.

Electron microscopy
Electron microscopy captures images at nanometer resolution, providing a complementary technique to overcome the low-resolution drawback of the epifluorescent experiments. However, dehydration of neurons, a critical step in transmission electron microscopy, could distort the native state of the membrane and organelles in fixed cells. Furthermore, the EM data show the status of the vesicles at the time of fixation, but not the entire time-lapse process of exocytosis or endocytosis. Therefore, this technique does not reflect how fusion and fission of vesicles naturally occur.

Therefore, combining electron microscopy and epifluorescence imaging, which can provide both high-resolution and live images of the endocytosis process, will create a more complete view of this cellular mechanism.

TROUBLESHOOTING
Problem 1
Low density and inferior quality of cultured neurons (steps 1–42).

Potential solution
To increase the yield of cell harvest, we will pay attention to the following details. 1) Dissection is always performed in an ice-cold solution. The low temperature of the solution is preserved by a frozen-cold metal block under the dish (Figure 1F); the metal blocks are precooled in the -20°C freezer for 2–3 h. The used metal block will be changed with a fresh one every 10–15 min. Also, all tissues in Petri dishes are temporarily stored on ice in case of degradation. 2) Dissection should be completed within about 1 h. 3) Triturate the hippocampal tissues only 2-3 times to prevent excessive cell death. If hippocampal tissue is collected from 6–10 pups, divide the tissue into 2 tubes and triturate them separately.

To protect the cell culture from contamination, we will pay attention to the following details. 1) Clean all surgery tools, pipette guns, tissue culture hood, and hands with 70% ethanol to minimize the risk of contamination. 2) Use a flame, instead of 70% ethanol, to sterilize the tweezers before using it to transfer the coverslips in plating (Step 19) and transfecting (Step 41) cells. 3) Use sterile dishes, culture plates, tubes, syringes, filters, and pipette tips. Never touch them with hands to maximize sterility. 4) Wear a mask when working with cells. 5) If a well is infected (medium changes in color from pink to yellow), remove all media from that well and leave it empty. Usually, neighboring wells will stay healthy if this step is followed. 6) In the incubator, plates with cultured cells are placed on top of empty plates to keep some distance between the plate with cultured cells and the shelf (Figure 3B). 7) Except for the Dissection Solution and HBSS (with Ca^{2+} and Mg^{2+}) used in the dissection, all solutions and media are filtered with 0.22 μm syringe filters. 8) To prevent unanticipated effects, antibiotics are not used in any media or solutions, except the Dissection Solution.

Glial cells are necessary for the development and growth of neurons in culture. If the number of glia cells is not controlled, they will inhibit neuron growth and make imaging experiments difficult. To control glial proliferation, add 2–3 μM Ara-C to the medium at DIV2–3. For example, 3 μM Ara-C is applied to the medium at DIV2 if the glial cells grow rapidly.

Problem 2

Unreliable data acquired from the epifluorescence imaging experiments (steps 48–57).

Potential solution

Reliable data depend on culture quality, transfection efficiency, and experimental conditions. The following issues are addressed to rule out any potential errors. 1) Control the intensity of the light source, room lighting, and the exposure time to minimize fluorescent bleaching. 2) Select at least 20 boutons for each experiment to minimize the variance of fluorescent signals among boutons. 3) Provide each of the selected boutons with its own background to make data normalization more accurate. (check the formula in Step 56). 4) Record at least 30 s of baseline before stimulation to determine if any changes result from the baseline shift due to photobleaching. 5) One coverslip can be used for one experiment. One culture can provide three or four coverslips. The number of experiments in one experimental condition, such as the wild-type group or mutation group, should be more than 10.

Problem 3

Low density of boutons under the electron microscope (steps 61–85).

Potential solution

Because of the small size of boutons (1 μm in diameter) and the higher magnification used in the EM experiments, a higher density of cells (4.0×10^5 cells/mL) is highly recommended in cell plating for these experiments.

More boutons in each thin section will make the EM study more effective. Preparation of the thin section containing more boutons requires experimenters with long-term training. For a short-term EM project, we recommend preparing the thin sections under the guidance of an EM expert.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ling-Gang Wu (wul@ninds.nih.gov).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

This study did not generate any new code. All data is available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

B.S. and X.-S.W. conducted the experiments, analyzed the data, and wrote the manuscript. N.C. participated in writing. M.S. conducted the EM experiments. L.-G.W. supervised the project and participated in writing. All authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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