# Supplemental Material

## Plasticity-Induced Growth of Dendritic Spines by Exocytic Trafficking from Recycling Endosomes

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### DNA Constructs

TfR-GFP was kindly provided by Gary Banker (Oregon Health Sciences University). Superecliptic pHluorin was a gift from George Augustine (Duke University). TfR-pHluorin was generated by replacing GFP in TfR-GFP with superecliptic pHluorin amplified by polymerase chain reaction (PCR). mRFP and tdTomato were provided by Roger Tsien (University of California at San Diego). TAT-syntaxin13ΔTM and TAT-syntaxin7ΔTM were generated by subcloning of syntaxin13ΔTM and syntaxin7ΔTM cDNAs from GFP-syntaxin13ΔTM and GFP-syntaxin7ΔTM (Park et al., 2004) into the pTAT vector which was kindly provided by Steven Dowdy (University of California at San Diego). GFP-Rme1-WT and GFP-Rme1-G429R cDNAs were provided by Frederick Maxfield (Cornell University Weill College of Medicine). GFP-Rab11a-WT was a gift from Marino Zerial (Max Planck Institute of Molecular Cell Biology and Genetics). GFP-Rab11a-S25N was generated from GFP-Rab11a-WT by site-directed mutagenesis (QuickChange, Stratagene) (Park et al., 2004). Syntaxin13-GFP was from William Trimble (University of Toronto). GFP-syntaxin13ΔTM construct was generated as described (Park et al., 2004). TAT-syntaxin13ΔTM and TAT-syntaxin7ΔTM were generated by subcloning of syntaxin13ΔTM and syntaxin7ΔTM cDNAs from GFP-syntaxin13ΔTM and GFP-syntaxin7ΔTM into the pTAT vector.

### Cell Culture and Transfection

COS-7 cells were grown in DMEM (Sigma) supplemented with 10% fetal bovine serum. Hippocampal neuron cultures were prepared from E18 rat embryos and maintained for 14-21 days *in vitro* as described (Ehlers, 2000). Neurons were transfected using either calcium phosphate or Lipofectamine 2000 (Invitrogen). For calcium phosphate transfections, DNA (1–2 μg) was mixed with 250 mM $CaCl_2$ and added to the same volume of 2X BES (50 mM BES, 280 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, pH 6.96). The DNA mixture was incubated for 20 min at room temperature and then gently dropped into the media. Approximately 8 hours after

transfection, neurons were washed with serum-free media and then incubated for 1-2 days. For transfection using Lipofectamine 2000, DNA (1–2 μg) in 25 μl serum-free Neurobasal media (Gibco) was mixed with 0.5 μl of Lipofectamine 2000 in 25 μl serum-free Neurobasal media that was pre-incubated for 5 min at room temperature. The DNA and lipofectamine mixture was incubated for 20 min at room temperature and added dropwise into neurons cultured in 12-well plates.

**Immunocytochemistry**

*His-TAT-Syn13ΔTM or His-TAT-Syn7ΔTM Staining.* Live hippocampal neurons were pre-incubated with TAT-Syn13ΔTM or His-TAT-Syn7ΔTM (3.5 μM each) for up to 2 hr at 37°C. Neurons were fixed with 4% paraformaldehyde/4% sucrose and then permeabilized with 0.1% Triton X-100 in PBS. Neurons were incubated with mouse anti-His (Calbiochem) for 1 hr at room temperature. Cells were washed and incubated with Alexa 488-conjugated secondary antibodies for 40 min at room temperature prior to visualization by fluorescent microscopy.

*Rab11 and Syntaxin13 Staining.* Neurons expressing GFP as a cell fill were fixed with 4% paraformaldehyde/4% sucrose in PBS and then permeabilized with 0.1% Triton X-100 in PBS. Neurons were incubated with anti-Rab11 (Zymed Laboratories Inc.) or anti-syntaxin13 (Synaptic Systems) for 1 hr at room temperature. Cells were washed and incubated with Alexa 568-conjugated secondary antibodies for 40 min at room temperature prior to visualization by fluorescent microscopy.

**Alexa-Tf Uptake to Label Recycling Endosomes**

Live neurons expressing GFP as a cell fill were incubated with Alexa 568-Tf (100 μg/ml; Molecular Probes) for 1 hr at 37°C to label recycling endosomes. Cells were then fixed in 4% paraformaldehyde/4% sucrose in PBS, and Alexa 568-Tf was visualized together with GFP by fluorescent microscopy.

**Purification of TAT-Syn13ΔTM and TAT-Syn7ΔTM**

Syntaxin13ΔTM and syntaxin7ΔTM cDNAs from pEGFP-syntaxin13ΔTM and pEGFP-syntaxin7ΔTM constructs (Park et al., 2004) were subcloned into the pTAT v2.1 vector and TAT fusion proteins were expressed in BL21 strain *E. coli* (Invitrogen). Briefly, overnight 100 ml cultures of BL21 cells containing the pTAT-Syntaxin13ΔTM or Syntaxin7ΔTM plasmid were

inoculated into 1 L culture media. Protein expression was induced with 0.5 mM IPTG for 3-4 hr. TAT-Syn13ΔTM and TAT-Syn7ΔTM proteins were purified by Ni-NTA affinity chromatography (Qiagen), subjected to ion-exchange chromatography on a Source 30Q column (Amersham Pharmacia Biotech), and desalted on a PD-10 G-25 sephadex column (Amersham Pharmacia Biotech).

**Labeling Purified TAT-Syn13ΔTM or TAT-Syn7ΔTM Proteins with Fluorescent Dyes**

Purified TAT fusion proteins (1 mg/ml) were labeled with Alexa488 using the Fluor 488 Protein Labeling Kit (Molecular Probes) for 1 hr at room temperature in the dark according to the manufacturer's instructions. Labeled protein mixtures were applied on a gel filtration column to separate Alexa-488-labeled TAT-Syn13ΔTM or Alexa-488-labeled TAT-Syn7ΔTM from unconjugated Alexa 488 dye.

**Transferrin Recycling Assay with TAT-Syn13ΔTM**

Live hippocampal neurons were pre-incubated with TAT-Syn13ΔTM (3.5 μM) or TAT-Syn7ΔTM (3.5 μM) for 1 hr at 37°C. Neurons were incubated with Alexa 488-Tf (50 μg/ml; Molecular Probes) in serum-free Neurobasal media containing TAT-Syn13ΔTM (3.5 μM) or TAT-Syn7ΔTM (3.5 μM) for 1 hr at 37°C to reach equilibrium. Neurons were then incubated in Neurobasal media with excess unlabeled Tf (5 mg/ml) but without TAT proteins for another 1 hr at 37°C. After washing, neurons were fixed and the remaining intracellular Alexa 488-Tf imaged.

**EGF Degradation Assay with TAT-Syn7ΔTM**

COS-7 cells were pre-incubated with TAT-Syn7ΔTM (3.5 μM) for 1.5 hr at 37$^{o}$C and starved in serum-free DMEM for 1 hr at 37$^{o}$C. Fluorescein-EGF (0.8 μg/ml; Molecular Probes) was added for 20 min at 37$^{o}$C. Cells were then washed and incubated in DMEM at 37$^{o}$C for another 20 – 40 min at 37$^{o}$C. Cells were then fixed and remaining intracellular fluorescein-EGF imaged.

**FM Uptake and Destaining**

For FM uptake experiments, presynaptic terminals were labeled by applying high $K^+$ HEPES-buffered solution (mM: 78.5 NaCl, 50 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 30 glucose, 25 HEPES, pH 7.4) in the presence of 10 μM CNQX, 50 μM AP5, 1 μM TTX, and 15 μM fixable FM4-64 (AM4-65; Biotium, Inc) for 1 min. Neurons were then washed with a HEPES-buffered solution (mM: 120

NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 30 glucose, 25 HEPES, pH 7.4) in the presence of 10 μM CNQX, 50 μM AP5 and 1 μM TTX for 10–15 min. Neurons were fixed with 4% paraformaldehyde/4% sucrose in PBS and imaged.

To measure FM destaining kinetics, presynaptic terminals were labeled by evoking presynaptic activity with extracellular electrical stimulation at 10 Hz for 60 seconds in a HEPES-buffered solution (mM: 120 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 30 glucose, 25 HEPES, pH 7.4) containing 10 μM CNQX, 50 μM AP5, and 15 μM FM4-64 (Molecular Probes). Neurons were then washed with the HEPES-buffered solution containing 10 μM CNQX, 50 μM AP5 and 1 μM TTX for 10–15 min. FM4-64 puncta were imaged every 20 seconds before and during field stimulation delivered at 15 Hz in HEPES-buffered solution containing 10 μM CNQX and 50 μM AP5.

**Live-Cell Imaging**

Twenty-four to 48 hours following transfection, neurons were imaged in a chamber (Dagan) filled with imaging buffer (mM: 120 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 15 glucose, 15 HEPES, pH 7.4) at 32° by heating the stage using an air stream incubator (Nevtek). Confocal images were acquired using a Yokogawa spinning disk confocal (CSU10, Solamere Technology Group), with excitation lines from a 2.5W KrAr laser (SpectraPhysics) selected and shuttered via an acousto-optical tunable filter (Neos Technologies) and emission directed through a filter wheel (Sutter Instruments) holding band-pass filters (Chroma). Images were acquired using a 60x Plan Apochromat objective (NA 1.4) and a 12-bit cooled CCD camera (Roper Scientific or Hamamatsu Inc.) and were analyzed using Metamorph (Universal Imaging Corporation).

For glycine stimulation, neurons were treated with glycine (200 μM) in $Mg^{2+}$-free extracellular solution (mM: 150 NaCl, 2 $CaCl_2$, 5 KCl, 10 HEPES, 30 glucose, 0.0005 TTX, 0.001 strychnine, 0.02 bicuculline methiodide, pH 7.4) for 3 min. Neurons were kept in extracellular solution without glycine until the end of imaging.

For acidic and ammonium chloride solution treatment during TfR-pHluorin imaging, acidic solution (pH 5.5) was prepared by replacing HEPES in the imaging buffer with MES (pH 5.5), with all other components in the imaging buffer remaining unchanged. Ammonium chloride solution (pH 7.4) was prepared by substituting 50 mM NaCl in the imaging buffer with $NH_4Cl$, with all other components remaining unchanged.

**Image Analysis and Quantification**

*Quantification of recycling endosome localization.* Cell-filled images (GFP for Figure 1C or mRFP for Figure 5C) and recycling endosome labeled images were thresholded at 1.21 to 1.86-fold (for cell-filled images) and at 1.35 to 2.18-fold (for recycling endosome-labeled images) over background. Regions created from the endosome image were then transferred to corresponding cell-fill images. Spines were defined by characteristic morphology in the cell-fill images. Endosome localization relative to spines was categorized according to the presence of an endosomal structure at the base (a), in the neck (b), or in the head (c) of spines as illustrated in Figures 1C and 5C. If a portion of the endosome region extended into the dendritic shaft as defined by a perpendicular line drawn at the base of the spine, it was also counted as being present at the base.

*Quantification of total protrusion, spine, and filopodia numbers.* Either mRFP or tdTomato channels of each image were scaled equally for counting total protrusions consisting of spines and filopodia. For morphological classification, spines were classified as protrusions < 2.75 μm in length with or without a head and filopodia were defined as unbranched protrusions of length > 2.75 μm without a spine head. For the quantification of Figure 3C, around 3,300 to 15,300 μm of dendritic length from 15 to 36 neurons each were analyzed. The n values correspond to the number of neurons analyzed. For quantification, the change in the total protrusion number was calculated by $N-N_0$ where N corresponds to the total protrusion number at each time point and $N_0$ indicates the averaged number of protrusions before application of glycine, AP5, Alexa488-TAT-Syn13ΔTM, or Alexa488-TAT-Syn7ΔTM as indicated. New filopodial protrusions from spine heads were counted as additional protrusions. All statistical analyses were performed using Student's t-test unless otherwise indicated. Between 1,200 and 1,500 μm of dendritic length was analyzed for each experiment in Figures 4C and 4D, where n values correspond to the number of neurons analyzed. Between 550 and 900 μm of dendritic length was analyzed for each experiment in Figures 3F, 3G, and 4E, where n values correspond to the number of separate dendritic segments from 3 to 5 neurons.

*Measuring TfR-pHluorin fluorescence during chemical LTP.* For measurements of TfR-pHluorin fluorescence during chemical LTP, the change in fluorescence (ΔF) was normalized to the averaged fluorescence intensity ($F_0$) before glycine treatment. ΔF was calculated by subtraction of $F_0$ from the measured fluorescence intensity at each time point. For analysis of dendritic shaft segments, dendritic regions within 0.9 μm of a spine outlet point were defined as

being "near spines" while dendritic regions > 0.9 μm away from all perpendicular lines drawn from all spine necks (including pre-existing and newly-formed spines) were defined as "away from spines".

*Measurement of spine or shaft area.* Cell-filled mRFP or tdTomato images were thresholded at 1.25 to 1.39-fold (for Figures 4G–4I, Figure 8, and Supplementary Figures S4 and S9) over background. Spine area (inclusive of spine head and spine neck) and shaft area were measured from maximum projected fluorescent images. For analysis of pre-existing spine growth, spines that existed throughout the imaging were selected. Normalized spine area was taken as the ratio of individual spine areas after stimulation (A) relative to the average spine areas before glycine stimulation or AP5 treatment ($A_0$). The absolute increase of spine size (ΔA) was obtained by subtraction of the spine areas before glycine application from the spine area after glycine stimulation.

**Electrophysiology on Cultured Hippocampal Neurons**

Whole-cell voltage clamp recordings were performed on DIV 17 – 24 rat hippocampal neurons cultured at high density on poly-lysine coated glass coverslips. Neurons were transfected with GFP (control), GFP-Rme1-G429R, GFP-Rab11a-S25N, or GFP-Syn13ΔTM cDNA constructs using Lipofectamine 2000 two days prior to recordings. Neurons were held at –60 mV using a MultiClamp 700A amplifier (Molecular Devices, Union City, CA) controlled from a workstation running MultiClamp Commander and pClamp software (Molecular Devices). The extracellular solution contained (in mM): 124 NaCl, 3 KCl, 1.25 $NaH_2PO_4$, 26 $NaHCO_3$, 20 D-glucose, 0.1 $MgCl_2$, 4 $CaCl_2$, 0.001 glycine, 0.05 picrotoxin, and 0.02 CNQX (330 mOsm/l, pH 7.3). Recording pipettes, with resistances between 3-5 MΩ, were filled with a solution containing (in mM): 30 Cs gluconate, 5 TEA-Cl, 3.7 NaCl, 20 HEPES, 0.3 Na-GTP, 4.0 Mg-ATP, 0.2 EGTA, 10 BAPTA, and 5 QX-314 (300 mOsm/l, pH 7.3). An extracellular stimulating electrode attached to a stimulus isolator (WPI, Sarasota, FL) was placed near the patched neuron and a 200 μsec 25 μA stimulus was applied initiated by Clampex 8.2 software. All currents were sampled at 10 kHz and low-pass filtered at 2 kHz and data were analyzed with Clampfit 8.2 software (Molecular Devices). NMDA receptor current decay time constants were obtained with single exponential fits to the data. All mean values are reported with standard errors of the mean (SEM).

**Slice Electrophysiology**

Five 15-day-old male Long-Evans rat pups were decapitated and their left hippocampi were dissected. Transverse hippocampal slices were chopped at 400 μm (Stoelting) at room temperature and then transferred to a static-pool interface chamber on nets glued to glass support rings positioned above ACSF (in mM): 117 NaCl, 5.3 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1 $NaH_2PO_4$, 26 $NaHCO_3$, 10 glucose, saturated with 95% $O_2$/5% $CO_2$. Slices were incubated at 32°C for one hour before two concentric bipolar stimulating electrodes (100 μm outer diameter, Fred Haer Co.) were placed 800 μm apart in CA1 stratum radiatum with a glass recording pipette positioned halfway in between, approximately 150-200 μm from the cell body layer. These distances assured activation of independent inputs by the two stimulating electrodes. Responses were amplified with an Axopatch 2B (Axon Instruments), filtered at 2 kHz, and recorded using customized IGOR software (Wavemetrics Inc.). The stimulus intensity was set at 75% of the baseline population spike threshold for the duration of the experiment. Test pulses were given to each input 30s apart every 120s, and a stable baseline was recorded on each input for at least 30 minutes at once per minute to each input. Saturating LTP was produced at one input by theta-burst stimulation (TBS), consisting of 10 bursts at 5 Hz of 4 pulses at 100 Hz given 8 times at a 30s interval (Abraham and Huggett, 1997; Staubli and Lynch, 1987), and LTP was monitored at the same stimulus intensity post TBS. The other input received control stimulation only.

**Serial Section Transmission Electron Microscopy (sSTEM)**

Slices were fixed at 5 min (n = 2 slices) or 30 min (n = 3 slices) after the delivery of TBS in mixed aldehydes (6% glutaraldehyde, 2% paraformaldehyde, 2 mM $CaCl_2$, and 4 mM $MgCl_2$ in 0.1M cacodylate buffer), and microwaved for 25 seconds to a final temperature of approximately 35°C (Jensen and Harris, 1989). Slices remained in fixative overnight at room temperature, were then rinsed in cacodylate buffer, and transferred to 0.1 M phosphate buffer. The area of CA1 containing the three electrode tracks was dissected out, embedded in 7% agarose, and sectioned perpendicularly through the slice at 70 μm on a vibrating blade microtome (Leica). Sections containing each stimulating electrode track plus the two surrounding adjacent sections were processed in a microwave oven (Ted Pella). Sections were post fixed under vacuum at 150W in chilled (19$^o$C) reduced osmium (1% $OsO_4$/2% KFeCN in 0.1 M cacodylate) for 4 minutes, and then in chilled 1% $OsO_4$ in 0.1 M cacodylate buffer for 4 minutes. After rinsing, sections were

dehydrated at 250W through a series of ethanol dilutions, each containing 1% uranyl acetate for 40 seconds. The ethanol was replaced with acetone, and the sections were infiltrated under vacuum at 350W with a series of acetone dilutions (50–100%) with LX-112 (Ladd) for 12 minutes total. The sections were embedded in flat coffin molds and cured at 60°C in a conventional oven for 48 hours.

All sectioning, photography, reconstruction, and analyses were done with the experimenter blind to condition. Coded resin blocks containing tissue sections that were adjacent to the sections with stimulating electrode indentations were used to obtain serial sections at the LTP and control sites. Sectioning trapezoids were positioned 50-80 μm lateral to the electrode indentations and 125-150 μm deep, for a diagonal distance of ~135-170 μm from the center of the stimulating electrodes. At this location the narrow PN15 dendritic arbor was not likely to have been stimulated directly, while all of the synapses were within range of presynaptic activation. These locations were at a depth of 175-200 μm from the air surface of the slice, an area where dendrites, synapses, and astroglia were well preserved and intact. Serial 45 nm sections were cut from each trapezoid on an Ultracut T ultramicrotome (Leica) and picked up on Pioloform-coated slot grids (Synaptek, Electron Microscopy Sciences). On average 100-200 serial sections were obtained from each sample and were imaged on a JEOL 1230 electron microscope with a Gatan 4K x 4K digital camera.

**sSTEM Reconstruction and Analysis**

Serial section images were aligned using the Reconstruct software developed in the Harris laboratory (by John Fiala, available at http://synapses.bu.edu). Lateral dendrites of comparable calibers were chosen for analysis. Membrane surface areas of spines and endosomal compartments were traced, and three-dimensional reconstructions and measurements were generated and rendered for publication by Reconstruct. The average length of the control and LTP dendritic segments was 8.5 μm (n = 14) and 8.0 μm (n = 14), respectively. There were 355 spines with 423 synapses, 24 nonsynaptic filopodia, and 52 RCs and 49 AVCs overall in both spines and dendritic shafts. In addition, there were 437 free endosomes (including small and large smooth or coated vesicles and segments of tubules) in spines. RCs, AVCs, and free endosomes were identified as previously determined in dendrites from PN15, PN21, and mature rat hippocampus that were perfusion fixed *in vivo* or in slices where gold colloid attached to BSA in the extracellular space was endocytosed into each of these compartments (Cooney et al., 2002).

None of these compartments were connected to the smooth endoplasmic reticulum, which did not have any BSA-gold particles and hence was not endosomal (Cooney et al., 2002).

**Immunogold Electron Microscopy**

Deeply anesthetized male Sprague-Dawley rats were perfused with saline followed by cold fixative, containing 4% paraformaldehyde, 0.1% glutaraldehyde, 0.1% picric acid in phosphate buffer (PB, 0.1M, pH 7.4). Brains were removed and post-fixed in the same fixative for 2 hours. Coronal sections (50 μm thick) were cut with a Vibratome, and then washed in PB. Floating sections were washed in phosphate buffered saline (PBS, 0.01 M, pH 7.4) and treated for 30 min in 1% sodium borohydride in PBS, washed in PBS, treated with 3% $H_2O_2$ in PBS, and again washed in PBS. Sections were then incubated in 20% normal donkey serum (NDS, Jackson ImmunoResearch) for 30 min, and overnight in mouse anti-TfR antibody (Zymed Laboratories Inc.) in PBS containing 2% NDS. Following several washes in PBS and incubation in PBS containing 2% NDS, sections were incubated in biotinylated donkey anti-mouse IgG (1:200, Jackson ImmunoResearch Laboratories Inc.) for 2 hours, washed with PBS, incubated with 1.4 nm gold particles conjugated to streptavadin (Nanoprobes), diluted 1:100 in PBS for 2 hours, and washed with PBS. Following gold labeling, sections were fixed with 1% glutaraldehyde (Electron Microscopy Sciences) in PBS for 10 minutes, and washed in PBS. Sections were washed in 0.05 M sodium acetate and gold particles were silver enhanced using IntenseS-EM (Amersham). After several washes in sodium acetate and PB, sections were post-fixed with 1% $OsO_4$ in PB for 45 min, washed in PB, washed in maleate buffer (0.1M), contrasted in 1% uranyl acetate in maleate buffer for 45 min, and washed in maleate buffer prior to dehydration in a graded series of ethanol washes. Ethanol was replaced by propylene oxide (Electron Microscopy Sciences) and the tissue was infiltrated with dilutions (50 – 100%) of Epon-Spurr's resin (6:4; Electron Microscopy Sciences) in propylene oxide over three hours. Following infiltration, sections were sandwiched between ACLAR films (Ted Pella), flattened between microscrope slides, and polymerized at 60°C for 48 hours. Chips from the area of interest (CA1) were removed and fixed to resin blocks for thin sectioning. Thin sections (~60 nm) were cut on a Leica ultramicrotome and collected on uncoated 200-mesh copper grids treated with Coat-Quick "G" (Electron Microscopy Sciences) for improved adhesion. Grids were stained with uranyl acetate and Sato's lead and examined in a Philips Tecnai electron microscope at 80 kV. Digital

micrographs were acquired with a Gatan 12 bit 1024x1024 CCD camera. Contrast of final images was adjusted using Adobe Photoshop.

**Supplementary References**

Abraham, W. C., and Huggett, A. (1997). Induction and reversal of long-term potentiation by repeated high-frequency stimulation in rat hippocampal slices. Hippocampus *7*, 137-145.

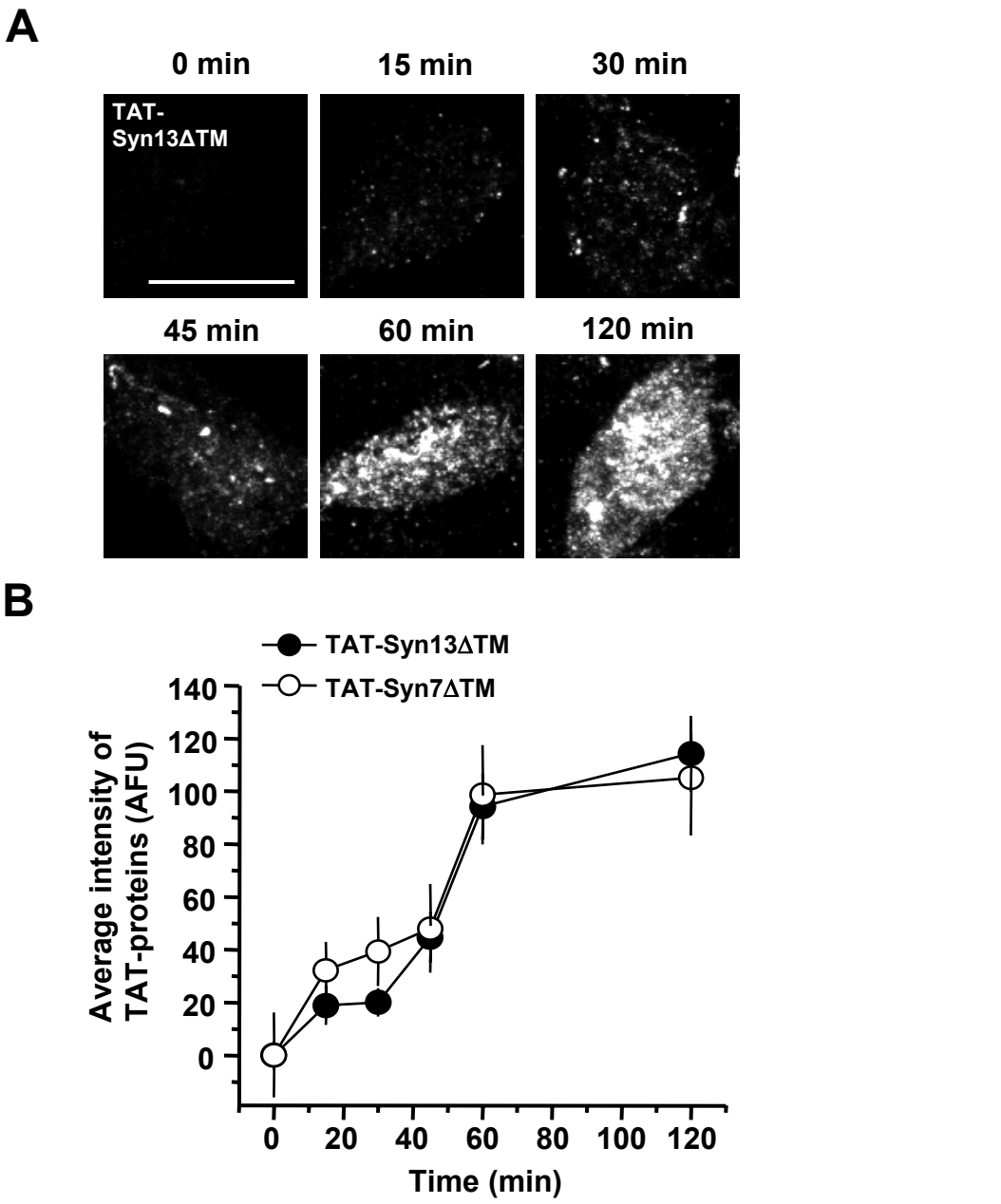
Cooney, J. R., Hurlburt, J. L., Selig, D. K., Harris, K. M., and Fiala, J. C. (2002). Endosomal compartments serve multiple hippocampal dendritic spines from a widespread rather than a local store of recycling membrane. J Neurosci *22*, 2215-2224.

Ehlers, M. D. (2000). Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. Neuron *28*, 511-525.

Jensen, F. E., and Harris, K. M. (1989). Preservation of neuronal ultrastructure in hippocampal slices using rapid microwave-enhanced fixation. J Neurosci Methods *29*, 217-230.

Park, M., Penick, E. C., Edwards, J. G., Kauer, J. A., and Ehlers, M. D. (2004). Recycling endosomes supply AMPA receptors for LTP. Science *305*, 1972-1975.

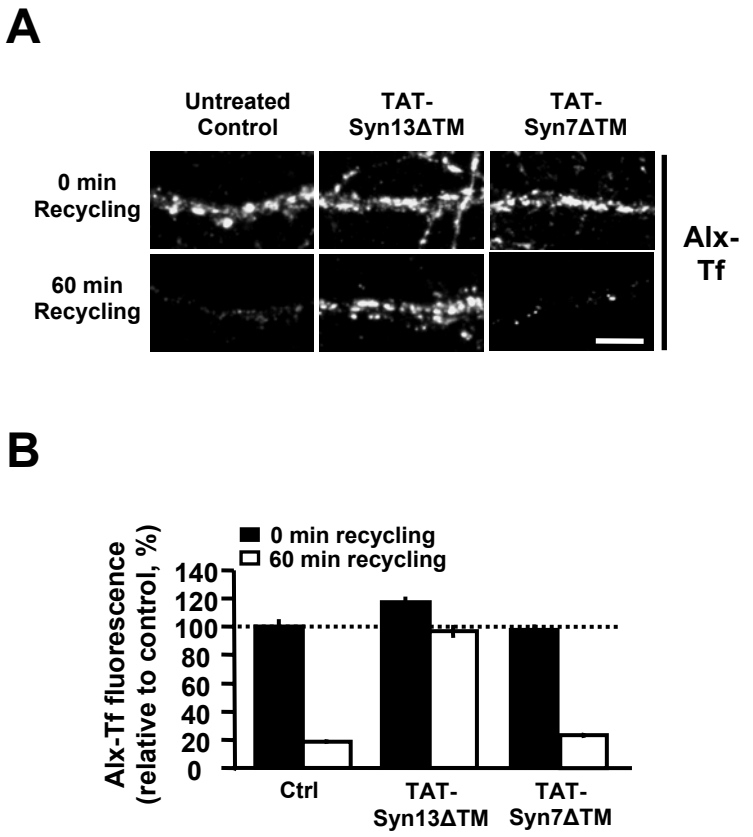
Staubli, U., and Lynch, G. (1987). Stable hippocampal long-term potentiation elicited by 'theta' pattern stimulation. Brain Res *435*, 227-234.



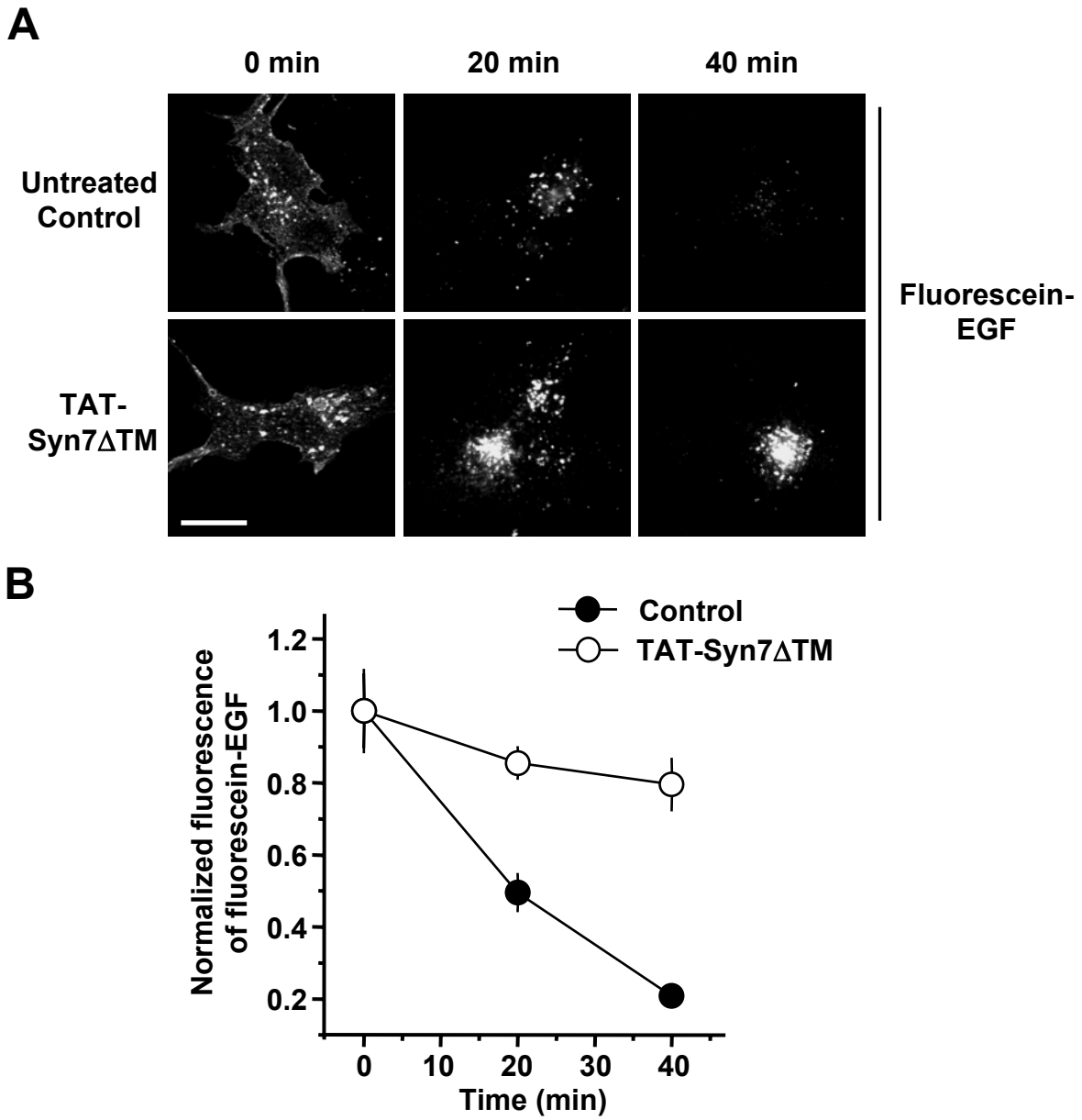

**Supplementary Figure S1.** Transduction Kinetics of TAT Fusion Proteins into Hippocampal Neurons

**(A)** His-tagged TAT-Syn13ΔTM (3.5 μM) was applied to hippocampal neurons (DIV17) for the indicated times. Neurons were fixed, permeabilized, immunostained by anti-His antibody, and imaged. Scale bar, 10 μm.

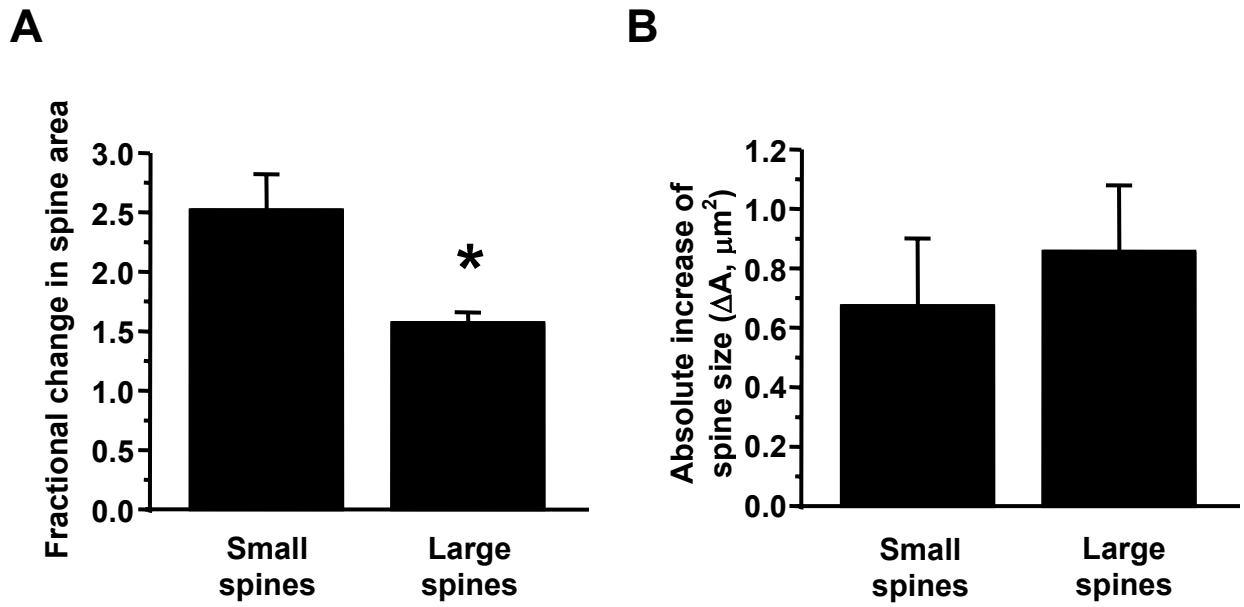
**(B)** Average fluorescence intensity of His-tagged TAT-Syn13ΔTM and His-tagged TAT-Syn7ΔTM that penetrated into neurons as shown in (A) was measured in the soma. n = 16, 17, 16, 20, 25, 19 for TAT-Syn13ΔTM and n = 12, 26, 19, 19, 21, 21 for TAT-Syn7ΔTM for 0, 15, 30, 45, 60, 120 min, respectively. Error bars indicate SEM.




**Supplementary Figure S2.** TAT-Syn13ΔTM Inhibits Recycling of Alx-Tf
**(A)** Neurons were pre-incubated with TAT-Syn13ΔTM or TAT-Syn7ΔTM proteins (3.5 μM each, 1 hr, 37 $^{o}$C). Alx-Tf uptake (1 hr, 37 $^{o}$C; 0 min recycling) and recycling (60 min recycling) were visualized by Alx-Tf fluorescence. Loss of Alx-Tf fluorescence after 60 min reflects recycling. Scale bar, 5 μm.
(**B**) Quantitative analysis of the remaining intracellular Alx-Tf from neurons treated with TAT-Syn13ΔTM, TAT-Syn7ΔTM proteins, or untreated control. n = 22, 22, 28, 24, 20, 19 from left to right. Data indicate means ± SEM.



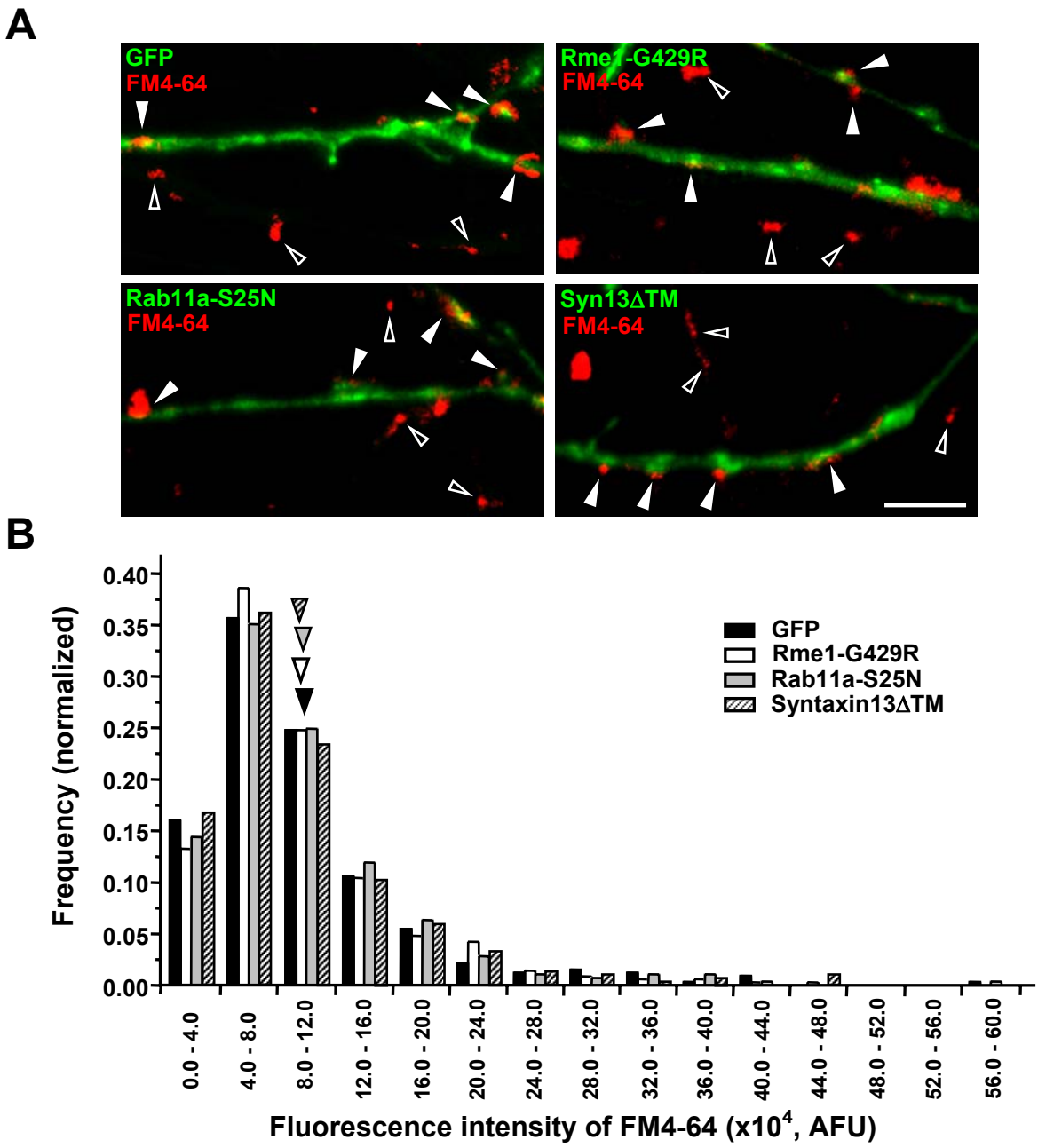

**Supplementary Figure S3.** TAT-Syn7ΔTM Inhibits Degradation of Fluorescein-EGF
(**A**) COS-7 cells were pre-incubated with control solution (top) or TAT-Syn7ΔTM (3.5 μM, 1.5 hr, 37°C) (bottom). After moving to serum-free media (1 hr, 37°C), cells were incubated in fluorescein-EGF (0.8 μg/ml) for 20 min at 37°C, washed, and subsequently incubated for the indicated times at 37°C prior to imaging. Loss of fluorescence reflects the degradation of fluorescein-EGF. Scale bar, 20 μm.
(**B**) Quantitative analysis of the remaining intracellular fluorescein-EGF from cells treated with TAT-Syn7ΔTM or untreated control. Data represent means ± SEM of fluorescein-EGF fluorescence intensity normalized to the average intensity at 0 min. Control: n = 25, 29, 31 for 0, 20, 40 min, respectively; TAT-Syn7ΔTM: n = 26, 26, 21 for 0, 20, 40 min, respectively.




**Supplementary Figure S4.** Small Spines and Large Spines Exhibit Different Fractional Increases but Similar Absolute Increases in Size Following Glycine Stimulation

**(A)** The fractional increase in spine size is greater for small spines. Spines analyzed before and after glycine stimulation (from Figure 4G) were subcategorized into small (<0.85 $\mu m^2$ projected area) and large (>0.85 $\mu m^2$) spines. Data represent means ± SEM of spine area at 12 min after glycine stimulation normalized to the average spine area before application of glycine as analyzed in Figure 4G. n = 9, 6; *$p < 0.05$; t-test.

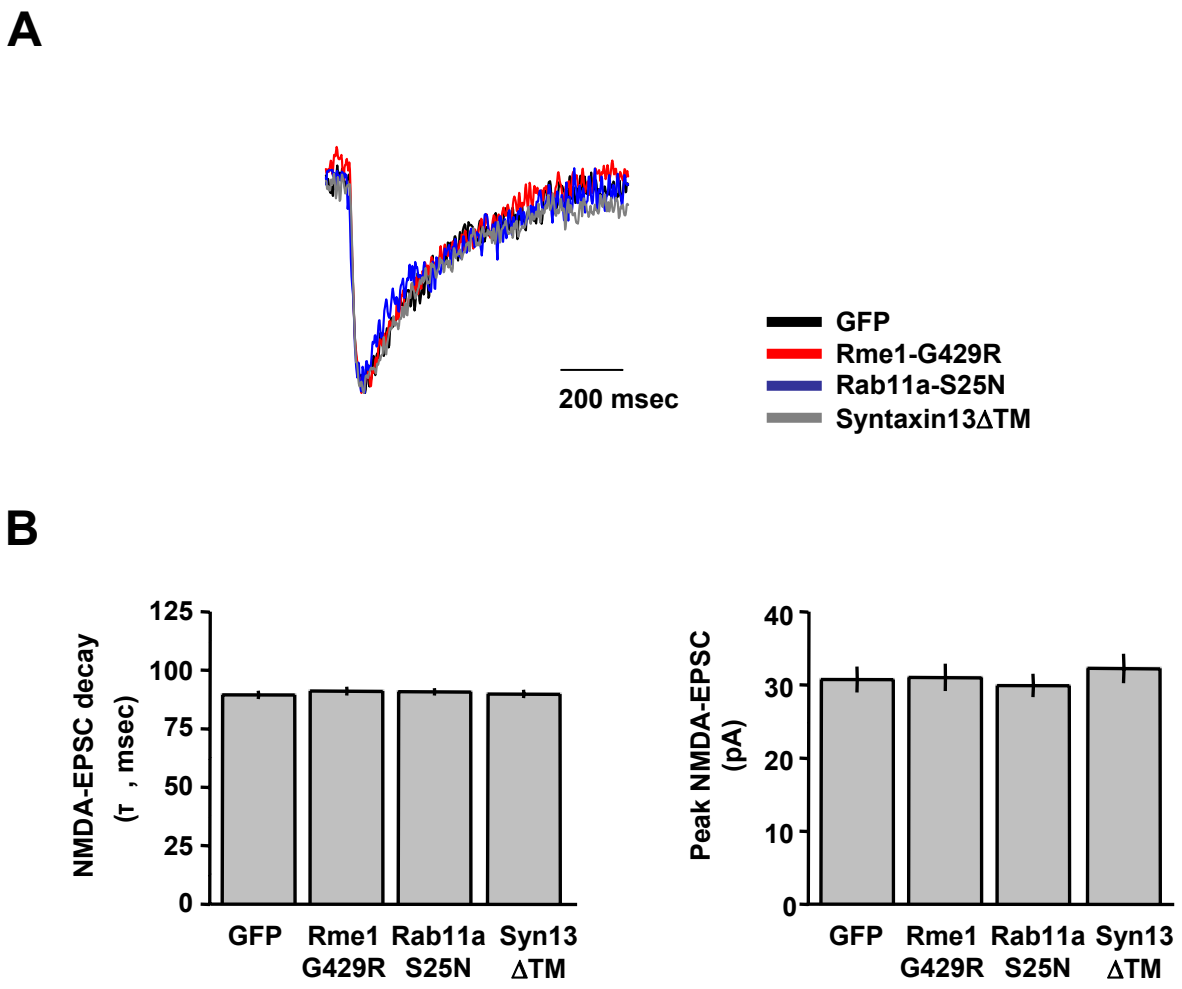
**(B)** The absolute increase of spine size is not significantly different between small and large spines. Spine areas before application of glycine were subtracted from the spine area at 12 min after glycine stimulation. Data represent means ± SEM. n = 9, 6; $p = 0.59$; t-test.




**Supplementary Figure S5.** Blocking Recycling Endosome Transport in Postsynaptic Neurons Does Not Affect Presynaptic FM Dye Uptake

**(A)** Presynaptic terminals onto hippocampal neurons expressing GFP, GFP-Rme1-G429R, GFP-Rab11a-S25N or GFP-Syn13ΔTM (DIV 16-17) were labeled by depolarization-induced uptake of FM4-64 (50 mM KCl, 15 μM FM4-64, 1 min; see Experimental Procedures for details). Filled and open arrowheads indicate FM4-64 puncta onto transfected and untransfected neurons, respectively. Scale bar, 5 μm.

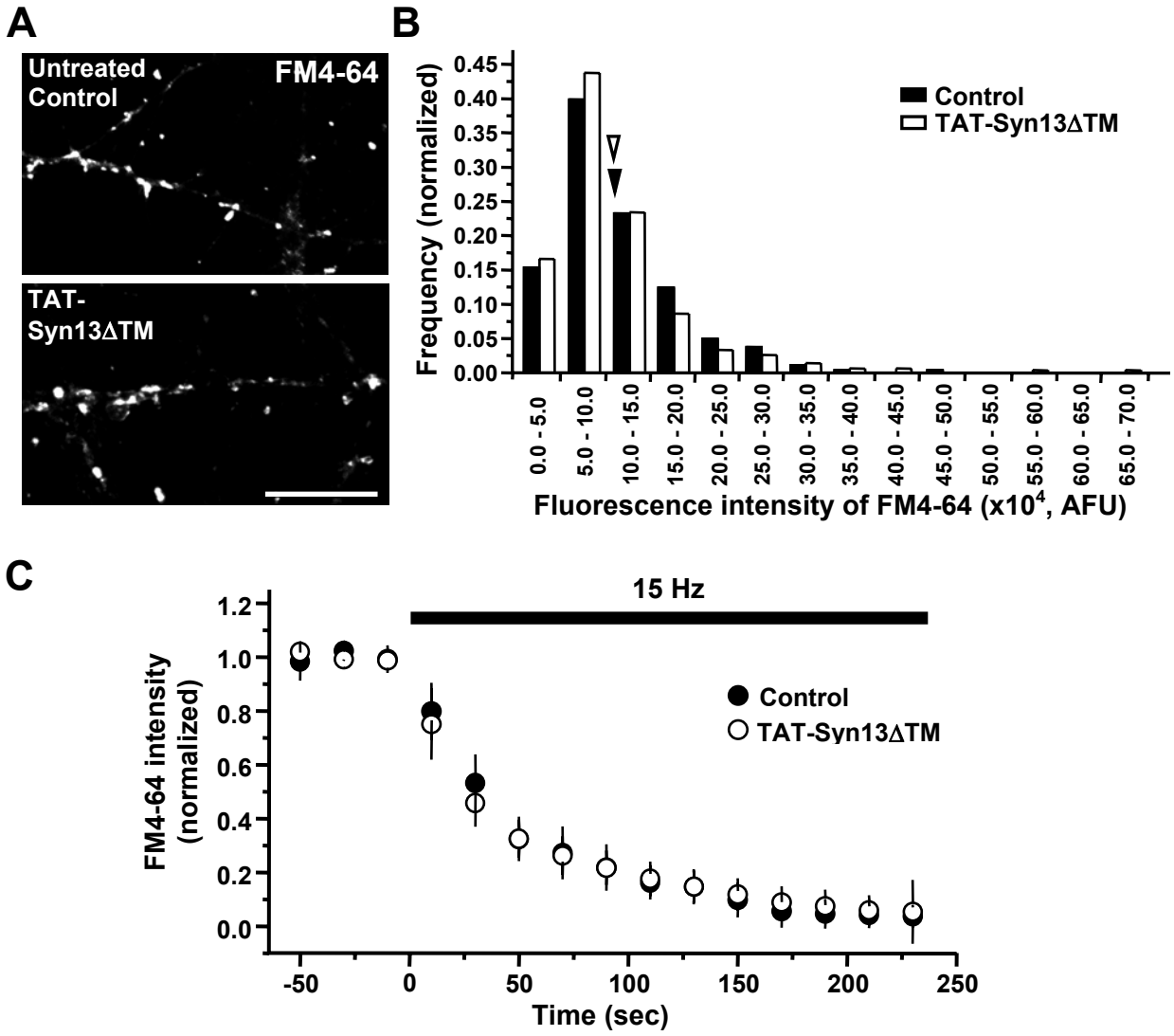
**(B)** Histogram representing the distribution of integrated fluorescence intensity values of individual FM4-64 puncta from (A). Arrowheads indicate mean values. AFU, arbitrary fluorescence units. n = 331, 355, 285, and 304 FM4-64 puncta from 7, 7, 10, and 8 neurons for GFP, Rme1-G429R, Rab11a-S25N, and Syn13ΔTM, respectively.




**Supplementary Figure S6.** Postsynaptic Perturbation of Recycling Transport Does Not Alter Evoked NMDA Receptor Currents in Cultured Hippocampal Neurons

**(A)** Overlaid normalized representative evoked NMDA receptor current traces from neurons expressing GFP (black), GFP-Rme1-G429R (red), GFP-Rab11a-S25N (blue), and GFP-Syntaxin13ΔTM (gray).

**(B)** Left panel, average decay rates (τ) of evoked NMDA receptor currents (GFP, 89.4 ± 1.7 msec; Rme1-G429R, 91.0 ± 1.8 msec; Rab11a-S25N, 90.9 ± 1.5 msec; Syntaxin13ΔTM, 89.8 ± 1.7 msec). Right panel, average peak NMDA receptor currents for GFP, GFP-Rme1-G429R, GFP-Rab11a-S25N, and GFP-Syntaxin13ΔTM (GFP, 30.7 ± 1.8 pA; Rme1-G429R, 31.0 ± 1.9 pA; Rab11a-S25N, 29.9 ± 1.6 pA; Syntaxin13ΔTM, 32.2 ± 2.0 pA). n = 19, 18, 18, and 17, respectively. Data represent means ± SEM.

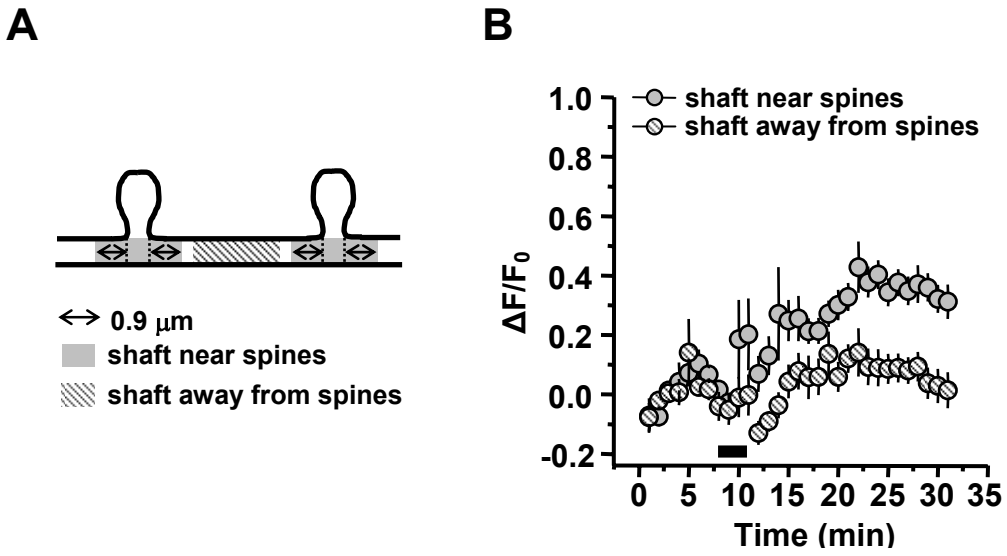



**Supplementary Figure S7.** Acute Application of TAT-Syn13ΔTM Does Not Affect Presynaptic Function

**(A)** Presynaptic terminals labeled by high $K^+$-induced uptake of FM4-64 in control neurons (top) or neurons treated with TAT-Syn13ΔTM (3.5 μM, 2 hr, 37°C) (bottom). Scale bar, 20 μm.

**(B)** Histogram of the integrated fluorescence intensity values for individual FM4-64 puncta. Arrowheads indicate mean values. AFU, arbitrary fluorescence units. n = 416 and 413 puncta from 5 and 6 neurons for untreated control and TAT-Syn13ΔTM, respectively.

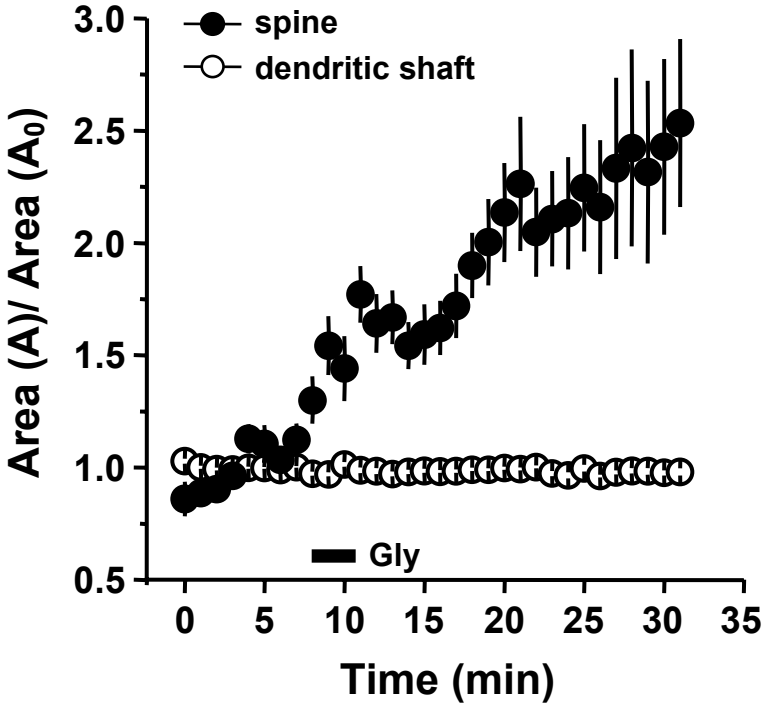
**(C)** Destaining time course of FM4-64 puncta in neurons treated with TAT-Syn13ΔTM or control solution in response to field stimulation. Following pre-incubation with TAT-Syn13ΔTM (3.5 μM, 2 hr, 37°C), presynaptic terminals were loaded with FM4-64 (15 μM) using field stimulation (10 Hz, 60 sec). After washout, FM4-64 puncta were imaged before and during the delivery of a 15 Hz train (black bar). Data represent means ± SD of the integrated intensity of individual FM4-64 puncta normalized to the average of initial three fluorescence intensities prior to destaining stimulation. n = 103 and 123 puncta from 5 and 8 neurons for control and TAT-Syn13ΔTM, respectively.




**Supplementary Figure S8.** Exocytosis From Recycling Endosomes in Dendritic Shafts Near Spines

**(A)** Dendritic regions within 0.9 μm (solid gray) of the base of spine necks or more than 0.9 μm away (black diagonal line) from any spine (new or pre-existing) were selected for analysis.

**(B)** Data represent means ± SEM of the fractional fluorescence increase ($\Delta F/ F_0$) of TfR-pHluorin from dendritic shaft segments near spines (dark circles; n = 10) and dendritic shaft segments away from spines (open circles; n = 9). The black bar indicates the period of glycine application. Note the ordinate scale difference compared to Figures 7C and 7E.




**Supplementary Figure S9.** Dendrite Shaft Size is Unchanged During Glycine-Induced LTP
Changes in the size of dendrites and spines following glycine stimulation in neurons expressing mRFP and TfR-pHluorin. Projected mRFP images were used for the analysis. Area (A) indicates the projected area of individual spines or dendrite segments at the plotted time. $A_0$ indicates the average area before glycine application. Glycine (200 μM) was applied during the times indicated by the black bar. n = 15 spines and 8 dendritic segments from 3 neurons each. Data represent means ± SEM.