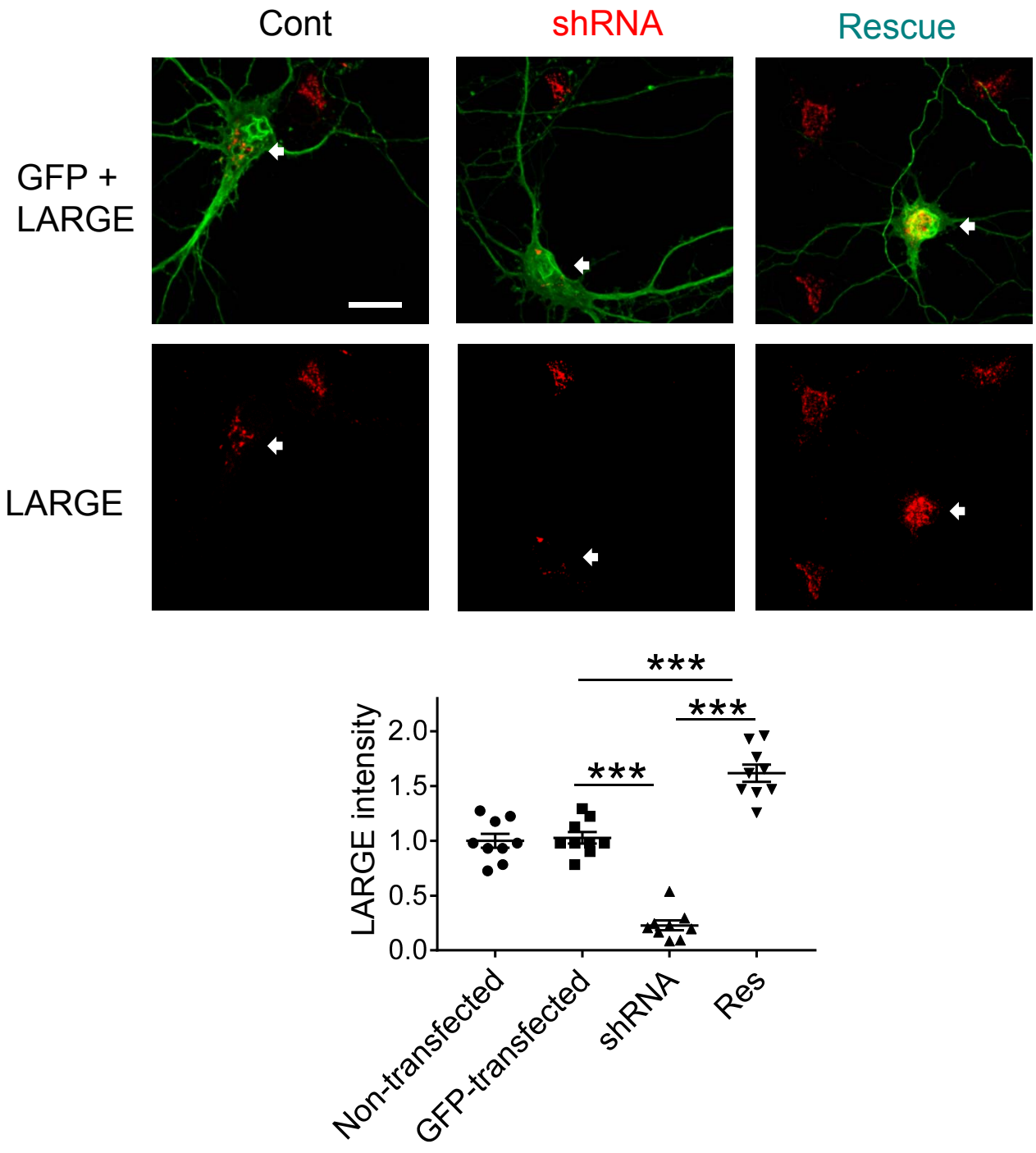
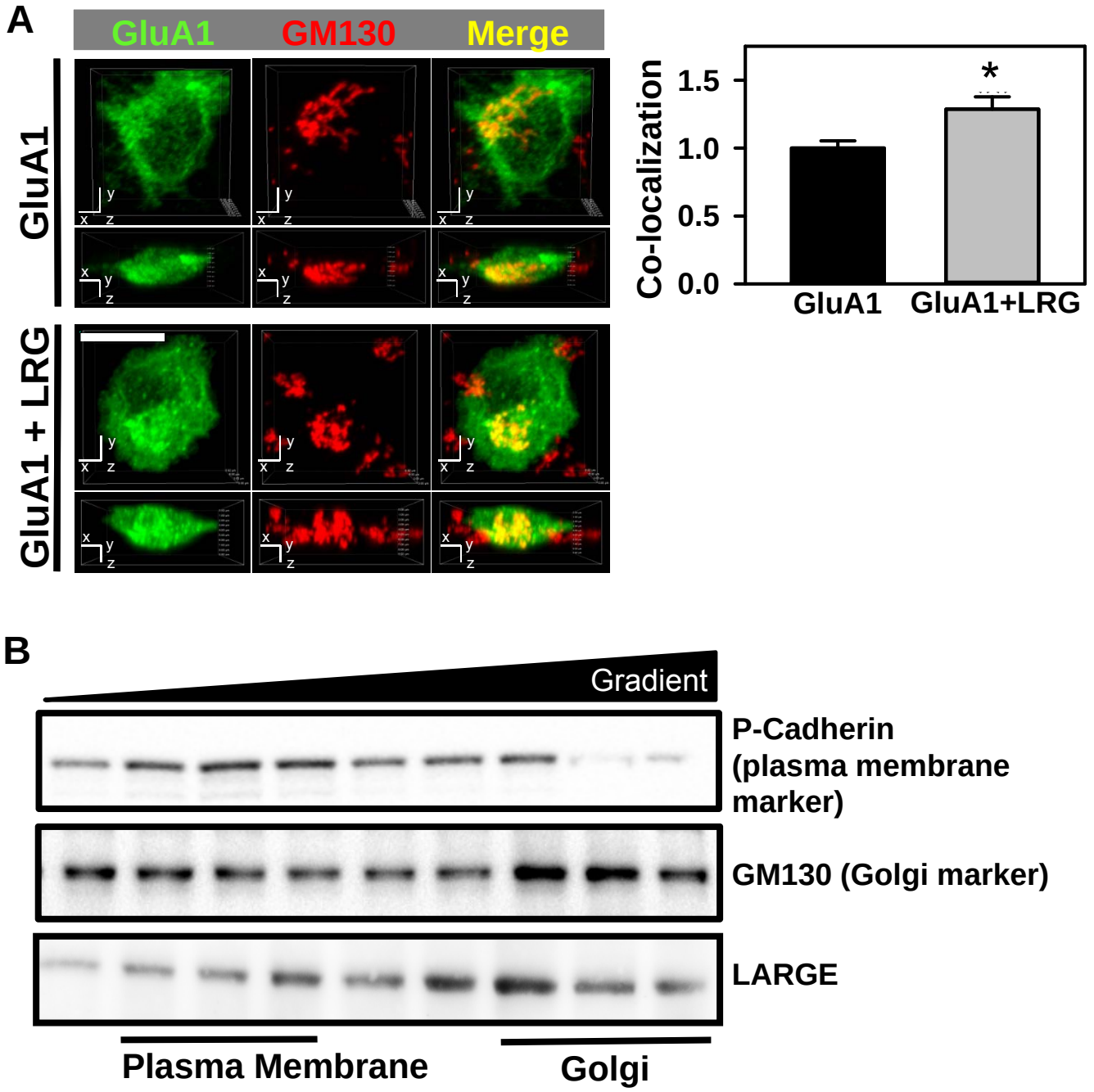
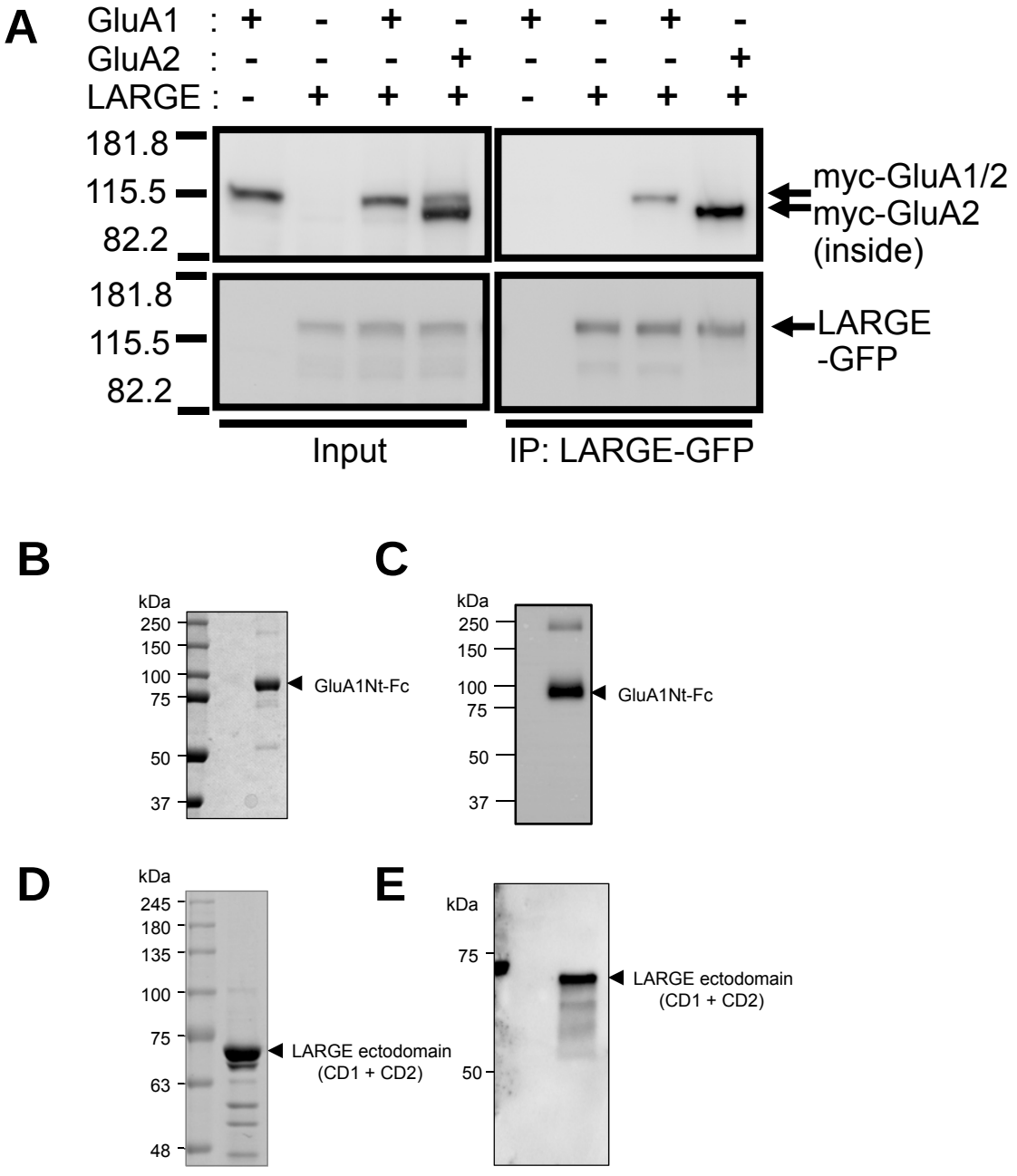


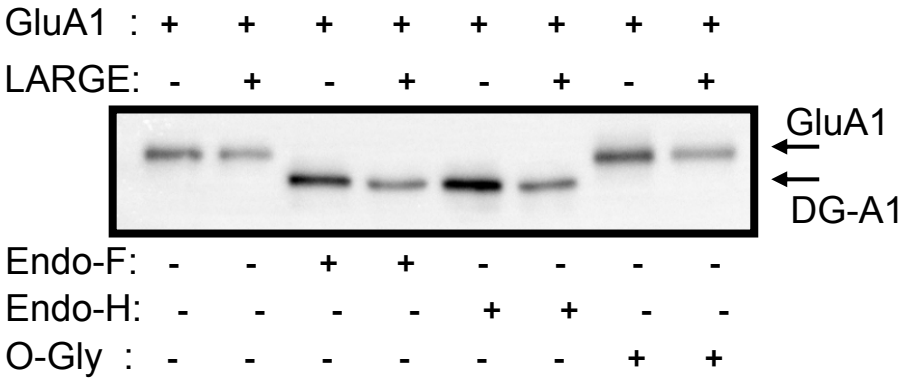
SI Appendix, Fig. S1. Validation of LARGE knockdown and rescue. Representative images show hippocampal cultured neurons transfected with scrambled shRNA with GFP (Cont), LARGE shRNA with GFP (shRNA) or shRNA and LARGE with GFP (Rescue). Quantification of *LARGE* knockdown indicated ~80% suppression of LARGE expression. LARGE rescue showed ~60% increase of LARGE expression. (n = 9, 9, 9, 9, respectively; one-way ANOVA, $F_{(3,32)}$=86.782, ****P* <0.001). Scale bar = 30 μm.



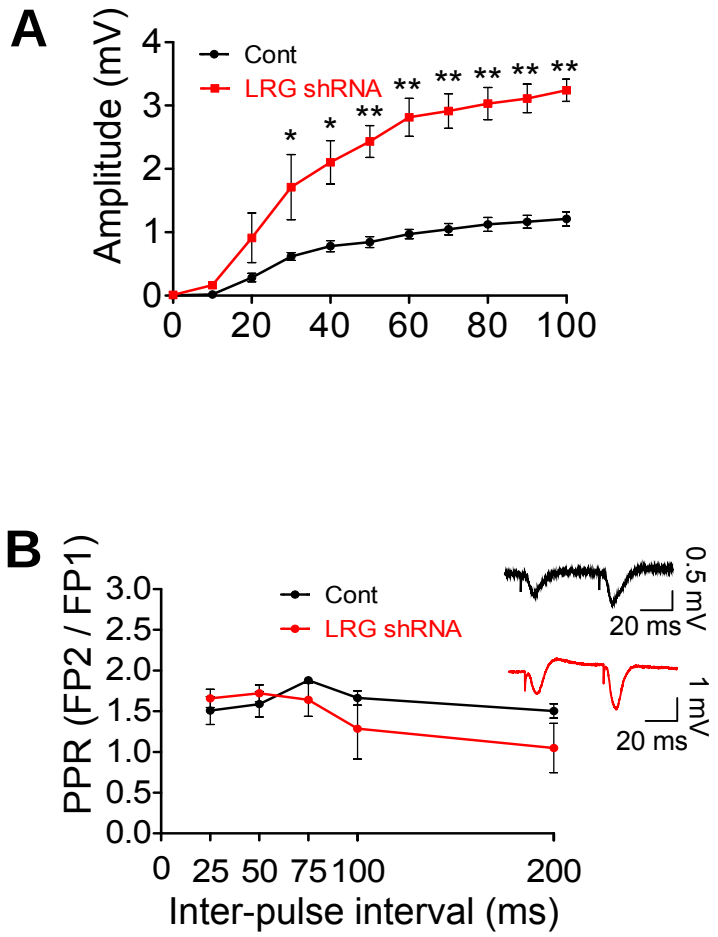
SI Appendix, Fig. S2. LARGE increased AMPA-R pool at the Golgi. (*A*) GFP-GluA1 at the Golgi was increased by LARGE overexpression (GluA1 + LRG), compared with a control (GluA1) (n = 30, 30 cells; Two tailed t-test, **P* < 0.001). Co-localization of GluA1 with GM130 in HEK293T cells analyzed by 3D reconstruction of a series of z-stack confocal images. Complete (yellow) and partial co-localization (orange). Co-localization of GluA1 and LARGE was quantified using the co-localization analysis tool in the NIS-Elements software (Nikon). In the analysis, Manders overlap coefficients were given and used to obtain the relative co-localization values between those two proteins. Scale bar = 10 μm. (*B*) Fractionation of subcellular organelles from the hippocampal CA1 of mice. Representative data showing the fractionation of organelle markers and LARGE. P-cadherin, plasma membrane marker; GM130, Golgi markers.



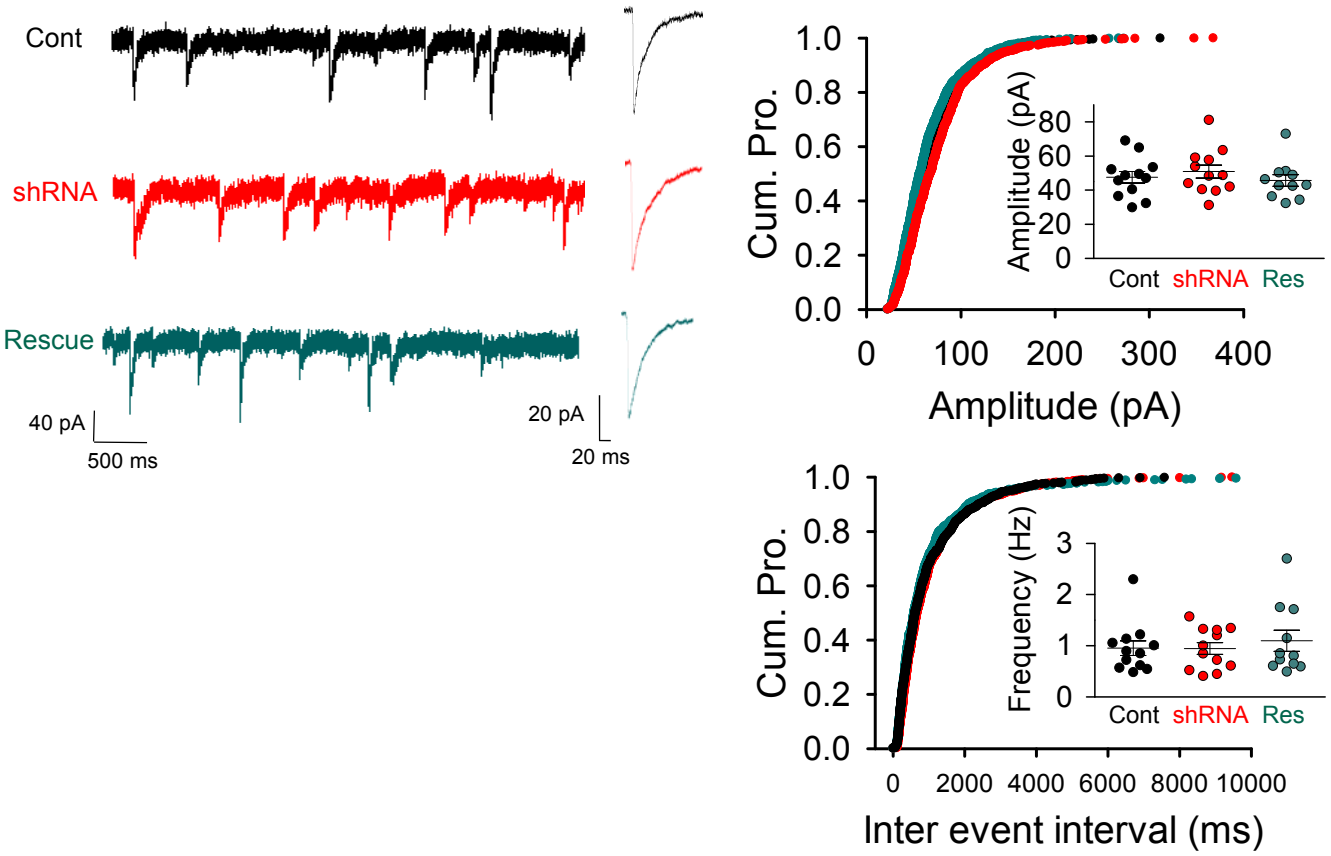
SI Appendix, Fig. S3. LARGE interacts with AMPA-R subunits. (*A*) LARGE interacted with GluA2 inside of cell (lower band) but not with GluA2 at the plasma membrane (upper band). Analysis of LARGE association with AMPA-R determined by immunoprecipitation (IP). HEK293T cells were transfected with myc-GluA1, myc-GluA2, and/or LARGE-GFP. IP with anti-GFP antibody. Both GluA1 and GluA2 were co-immunoprecipitated with LARGE-GFP. As see in Input, GluA2 yield two bands (upper and lower bands). Most GluA2 co-immunoprecipitated with LARGE was GluA2 correspond to intracellular GluA2, judging from its molecular weight. (*B*-*E*) Purification of GluA1 and LARGE (catalytic domain 1 [CD1] + CD2) proteins. SDS-PAGE (*B*) and Western-blot (*C*) analysis of purified GluA1Nt-Fc fusion protein. SDS-PAGE (*D*) and Western-blot (*E*) analysis of purified LARGE.



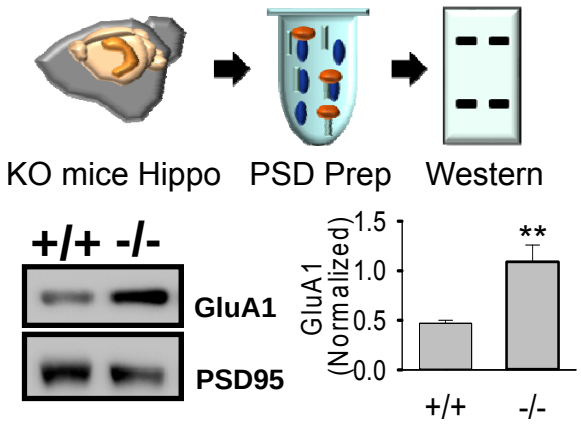
SI Appendix, Fig. S4. LARGE interaction with AMPA-R did not change glycosylation of AMPA-R. No change in N- and O-glycosylation of GluA1 with co-expression of LARGE in HEK293T cells. N-linked glycosylation of GluA1 was analyzed using two de-glycosylation enzymes, endoglycosidase F (Endo-F) and endoglycosidase H (Endo-H). Endo-F completely de-glycosylated all GluA1 (DG-A1) regardless of LARGE co-expression, and there was no difference in the amount of Endo-H–sensitive and –insensitive forms of GluA1 with co-expression of LARGE. In addition to N-linked glycosylation of GluA1, O-linked glycosylation of GluA1 was analyzed using O-glycosidase (O-Gly). The size of a GluA1 band was not changed by O-Gly treatment, suggesting that GluA1 does not have O-glycosylation. Co-expression of LARGE did not changed the O-glycosylation status of GluA1. Method: In HEK293T cells, myc-GluA1 and LARGE were expressed by transfection of their plasmids. From the lysate of the cells, myc-GluA1 was immunoprecipitated and treated with Endo-F (500 unit) and Endo H (500 unit) at 37℃ for 2 hours, or with O-Gly (50000 unit) followed with SDS-PAGE and Western blot analyses.



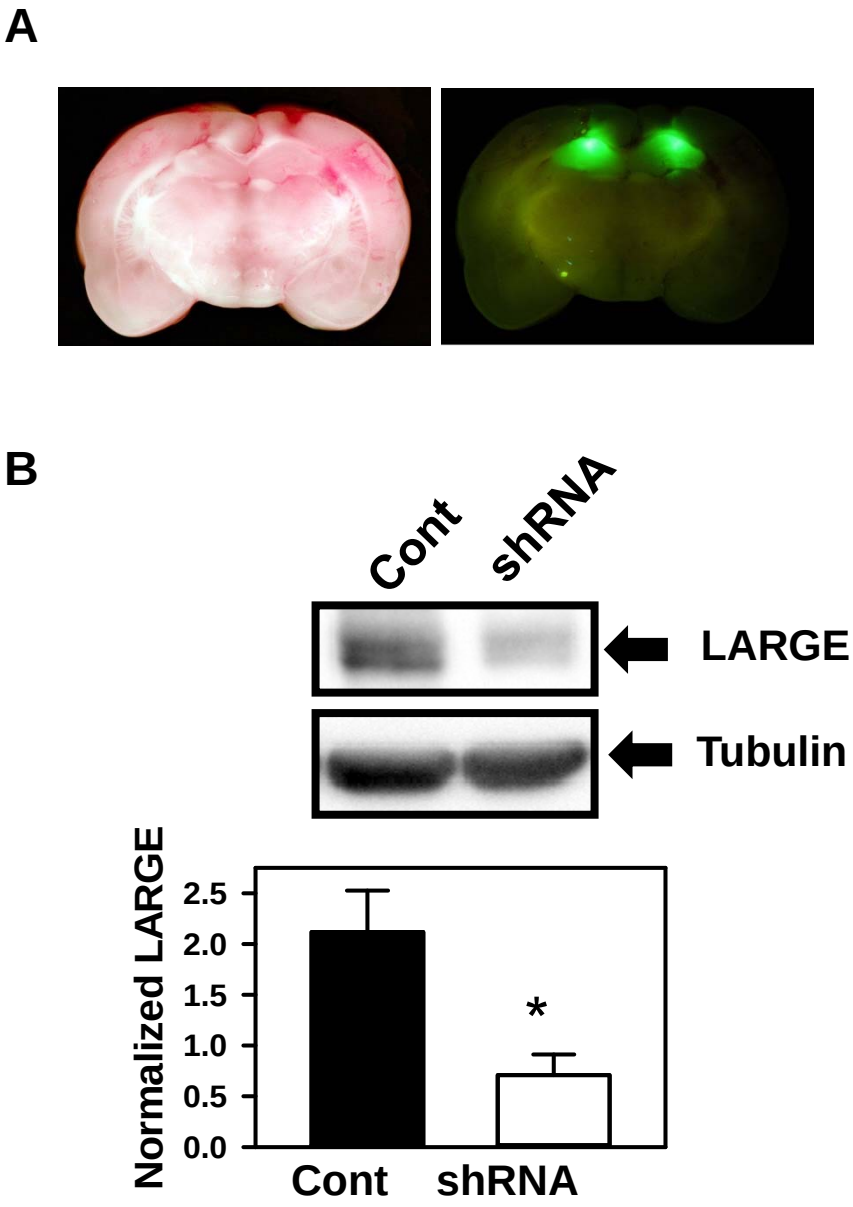
SI Appendix, Fig. S5. Electrophysiological analyses *in vivo* after knockdown (KD) of *LARGE* at CA1 of hippocampus. (*A*) AMPA-R fEPSP amplitudes were significantly increased by LARGE KD (LRG shRNA). Input-output curves shown relating stimulus strength to fEPSPs (output amplitude), which is greater in a LRG shRNA group (n = 5, 5 mice; Two-way repeated-measures ANOVA with post hoc Bonferroni t-test, *P < 0.05, **P < 0.001). (*B*) Intact short-term plasticity. Mean paired-pulse ratio (PPR) (FP2/FP1) of fEPSPs plotted as a function of inter-pulse. Field potential (FP) data are mean ± S.E.M. (group: P = 0.565, group x interval: P = 0.170, n=5, 5).



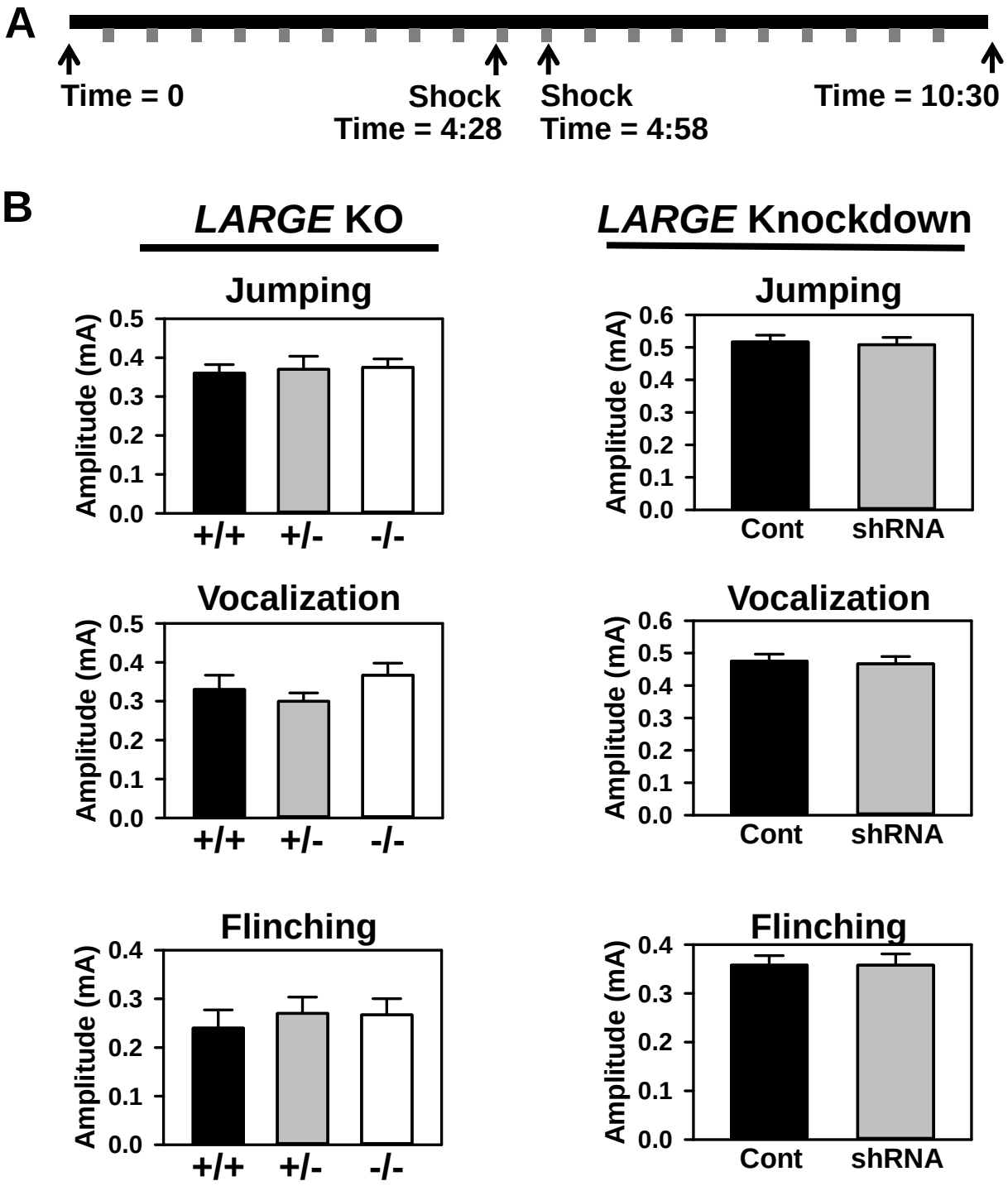
SI Appendix, Fig. S6. LARGE did not affect inhibitory synaptic strength. Miniature inhibitory postsynaptic current (mIPSC) recordings from Cont, shRNA, and Rescue. No significant differences in mIPSC amplitude and frequency were observed among the groups. Three different groups were recorded using 27 coverslips in 3 batches of neuronal culture (1098, 1080, 1055 events from n = 12, n=12, n=11 neurons, respectively; Amplitude, $F_{(2,32)}$=0.553, *P* =0.58; Frequency, $F_{(2,32)}$=0.292, *P*=0.749).



SI Appendix, Fig. S7. Schema, blot, and bar graph of a western blot analysis of GluA1 expression in the postsynaptic density (PSD) of hippocampi from LARGE knockout (KO) mice (-/-), demonstrating increased synaptic AMPA-R expression relative to that observed in WT mice (+/+) *in vivo* (n = 3, 3 mice; two-tailed t-test, **P <0.01).



SI Appendix, Fig. S8. Digital image and western blot analysis of hippocampal CA1 region of rat brain infected with AAV. (*A*) Brain slices were imaged by digital imaging under a blue LED light. Strong and specific expression of GFP in hippocampi indicated specific delivery and expression of LARGE shRNA with GFP by AAV injection. (*B*) Western blot analyses confirmed knockdown of LARGE in GFP-expressing hippocampi (n = 3). The LARGE signal was normalized to that of tubulin and averaged for quantification. Statistical analyses were performed using Student's t-test. *P < 0.005.



SI Appendix, Fig. S9. The absence of a significant difference in shock threshold among groups demonstrated that LARGE expression status did not affect fear conditioning. (*A*) Schema of shock threshold tests of animals. Shocks (gray blocks) were delivered every 30 s, with intensity increasing from 0.1 mA to 1.0 mA (4:28, 4:58) and decreasing back to 0.1 mA (10:30). (*B*) Regardless of genotype or injected AAV, all animals responded to shocks in a similar way. The shock intensity thresholds for jumping, vocalization, and flinching were measured for wild type (+/+), heterozygous (+/-), and knockout (-/-) animals and animals injected with AAV expressing scrambled shRNA with GFP (Cont) or *LARGE* shRNA with GFP (shRNA).

SI Methods

Animal care and treatments

Experiments involving animals were performed in accordance with procedures approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch (UTMB) and the Korea Advanced Institute of Science and Technology (KAIST). Care was taken to minimize the number of animals used and their discomfort. Colonies of *Large*^{myd} mice originated from Jackson Laboratory and were transferred from Dr. Kevin Campbell's laboratory at the University of Iowa prior to establishment at UTMB. Male adult C57BL/6 mice (wild type) were used in this study. The Sprague–Dawley rats and C57BL/6 mice used for behavior tests and neuronal cell culture were purchased from Harlan (USA) or Orient (Korea).

Neuronal culture

Hippocampal neuronal cultures were prepared and maintained with glia-conditioned media as previously described (1). Briefly, timed pregnant female C57BL/6 mice and Sprague–Dawley rats were purchased, and primary cells were isolated from embryonic day 17–18 (E17–18) pups. Mixed cell cultures containing both neurons and glia were then grown on coverslips in 12-well plates and in 6-well plates for use in biochemical experiments. The cultures were treated with 5 μM cytosine β-D-arabinofuranoside (AraC, Sigma) at day in vitro (DIV) 3 to reduce the number of glial cells.

HEK293T cell culture

HEK293T cells were cultured at 37ºC in high-glucose Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% fetal bovine serum (Gibco) and antibiotics (Gibco). For transfection, cells were plated on coverslips in 12-well plates or 6-well plates and then transfected with various expression plasmids using the transfection reagent TransIT-X2 (Mirus #mir6000).

Preparation of cDNA, shRNA, virus, transfection

The LARGE cDNA plasmid was kindly provided by Dr. Kevin Campbell (University of Iowa, Iowa City, IA, USA) and has been described previously (2, 3). The shRNA virus constructs were designed, generated, and screened as previously described (1). Briefly, four siRNAs were designed using Custom *SMART*pool Design and synthesized (GE Dharmacon). The KD efficacy of each siRNA was tested, and the sequence of the selected siRNA (rat: CGGCUUUGCUGCCUUGAAA, mouse: UGGCUUUGCUGCCUUGAAA) was used to design an shRNA. The *LARGE* rescue construct was generated by replacing the sequence encoding GFP in the pAAV vector used for *LARGE* KD with a *LARGE* cDNA containing a shRNA-resistant sequence (CGGCUUUGCUGCCUUGAAA => CGGCUUUGCUGCCCUGAAA).

The AAV was packaged and purified as follows. The shRNA designed from the selected siRNA sequence was subcloned into the pAAV vector, and subsequently packaged into the virus by co-transfecting HEK293T cells with pHelper and pAAV-RC (serotype DJ/8). At 72 h post-transfection, the viral particles were harvested through two freeze/thaw cycles and sonication. Benzonase and Rnase I were added to the virus-released solution. To remove cell debris, the cell lysates were centrifuged at 2500 × g for 15 min, and the supernatants were filtered through 0.2-μm syringe filters (Millipore, USA). A stock solution of 2.5 N NaCl and 40% PEG8000 (Sigma #5413) was added to the supernatant to yield final respective concentrations of 0.5 N and 8%. The resulting solution was incubated on ice for 3 h and centrifuged at 2000 × g for 30 min, after which the supernatant was discarded. The pellets containing AAV were resuspended in HEPES buffer, and this crude AAV solution was treated with chloroform and PEG for an aqueous two-phase extraction (10% PEG8000–13.2% $(NH_4)_2SO_4$) and final dialysis. The titer (>1 $\times$ 10^{11} TU/ml) was measured by treating 10^6 neurons with the AAV and measuring enhanced green fluorescent protein (GFP) expression after 1 week. The KD efficacy of AAV was evaluated using western blotting (Fig. 2*A* and S8*B*).

Cell transfection procedures used the same shRNA constructs (pAAV) except for the rescue construct. For these experiments, the *LARGE* rescue construct was generated by adding a self-cleaving 2A peptide (P2A) site between the sequence encoding the *LARGE* cDNA containing the shRNA-resistant sequence and enhanced GFP within the *LARGE* shRNA-containing pAAV vector. The KD and rescue efficacies were evaluated using confocal imaging.

Fractionation of subcellular organelles from cells or tissues

The iodixanol-based iso-osmotic density gradient-based subcellular organelle fractionation procedure was developed according to the instructions of the kit manufacturer (OptiPrep, Sigma #D1556), and optimized for two scales (5-ml volume gradient and 0.7-ml volume gradient). Briefly, cells or brain lysates were centrifuged for 10 min at 3000 × g, and the pellet was discarded. The supernatant was then centrifuged for 1 h at 100,000 × g to remove cytosolic contamination. The resulting second pellet was applied to the top of an OptiPrep discontinuous iodixanol gradient formed by the stepwise addition of solutions with increasing percentages of iodixanol (diluted in PBS). The 5.0-ml volume gradient was formed by adding 0.385 ml of 2.5%, 0.77 ml of 5%, 0.77 ml of 7.5%, 0.77 ml of 10%, 0.192 ml of 12.5%, 0.77 ml of 15%, 0.192 ml of 17.5%, 0.192 ml of 20%, and 0.192 ml of 30% iodixanol solutions to the bottom of a 5-ml Beckman centrifuge tube. The 0.7 ml volume gradient was formed by adding 27.5 µl of 30%, 27.5 µl of 20%, 27.5 µl of 17.5%, 110 µl of 15%, 27.5 µl of 12.5%, 110µl of 10%, 110 µl of 7.5%, 110 µl of 5%, and 55 µl of 2.5% iodixanol solutions to the bottom of a 750-µl Beckman centrifuge tube. After centrifugation for 2 h at 150,000g in a SW55i rotor and Beckman Optima centrifuge, 18–25 fractions were collected from the top of the column, depending on the experimental condition. Proteins in the fraction sets were resolved by SDS-PAGE, followed by western blot analyses with antibodies against the proteins of interest, including organelle markers, LARGE, and GluA1. The proteins were quantified by the densitometric analysis of bands in digital images of western blots and subjected to statistical analyses. For each protein, the average density in each fraction was normalized to the peak

density of the protein and plotted as a line graph. For organelle markers, each group was initially analyzed separately. However, as no significant changes were observed between wild-type and mutant *Large*^{myd} mice, the data were subsequently merged into a single group.

Immunoprecipitation

Small-scale immunoprecipitation was performed as described previously (4), with some modifications.

Protein expression and purification of LARGE and Fc-fused GluA1Nt, 2Nt, and 4Nt for ELISA

The gene encoding the human LARGE catalytic domain (CD1–CD2) was cloned into a pET21a expression vector (Novagen) using the *NdeI* and *XhoI* restriction sites. The resulting vector was transformed into Origami B (DE3) host cells (Novagen) to facilitate the formation of disulfide bonds. Single colonies were seeded into LB media supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), and tetracycline (15 μg/ml). After an overnight incubation, 10 ml of cultured cells were inoculated into 1,000 ml of fresh LB media. Once the cell density at 600 nm reached approximately 0.5, isopropyl-d-1-thiogalactopyranoside (IPTG) and $MnCl_2$ were added to final concentrations of 0.1 mM and 0.2 mM, respectively, to induce protein expression. The induced cells were further cultured at 18ºC for 3 days. Following a cell harvest via centrifugation at 6,000 rpm, the cell pellet was resuspended in lysis buffer (20 mM Tris, 150 mM NaCl, 10 mM imidazole, pH 8.0) and subjected to disruption by sonication. The sonicated lysate was subjected to ultracentrifugation at 13,000 rpm and 4ºC for 1 hour, and the supernatant was filtered through a 0.2-μm syringe filter (Millipore) and incubated with His-bind agarose resin (Elpis Biotech, Korea). After washing with a washing buffer (20 mM Tris, 150 mM NaCl, 20 mM imidazole, pH 8.0), LARGE proteins were eluted using an elution buffer containing 200 mM imidazole. Purified LARGE was subjected to a buffer change to a Tris-based buffer (20 mM Tris, 150 mM NaCl, pH 8.0) supplemented with 0.2 mM $MnCl_2$. This solution was stored at 4ºC for further study.

Fc-GluA1Nt, 2Nt, and 4Nt were purified from HEK293T cells using a transient transfection protocol. Cells were cultured in 10 cm x 15 cm plates to 85–90% confluency and transfected with the target vectors (10 μg/plate). At 72 h post-transfection (Mirus), the cells were harvested via centrifugation at 6,000 rpm, and the cell pellet was resuspended in PBS and lysed by sonication. The lysate was then subjected to ultracentrifugation at 13,000 rpm and 4ºC for 1 hour, and the supernatant was filtered through a 0.2-μm syringe filter (Millipore) and incubated with Protein A Sepharose beads (GE Healthcare #17-0780-01). After washing with a washing buffer (0.5% Triton X-100, 0.5 mM EDTA, 0.5 mM in PBS), the target proteins were eluted using an elution buffer containing 0.2 M glycine (pH 2.5). A proteinase inhibitor was added to all purification steps. Purified LARGE was then subjected to a buffer change using a Tris-based buffer (20 mM Tris, 150 mM NaCl, pH 8.0) and stored at 4ºC.

Enzyme-linked immunosorbent assay (ELISA)

A 96-well plate (SPL, Korea) was coated with purified LARGE protein and bovine serum albumin (BSA) at 4ºC. The following day, the antigen-coated plate was washed with PBS (pH 7.4) three times, and each well was incubated with a blocking buffer (PBS containing 0.1% Tween-20 and 2% BSA; PBST-BSA) at room temperature for 1 hour. All buffers used in this study were supplemented with 2 mM $MnCl_2$. After three washes with PBST, 10 μg/ml of Human-Fc fused GluA1 (GluA1-Fc) was added to the wells and incubated at room temperature for 1 hour. To detect GluA1 binding to the LARGE-coated surface, the plate was washed and incubated for 1 hour with goat anti-Human IgG (Fc specific)−Peroxidase (Sigma #A0170) diluted 1:3,000 in PBST-BSA. Binding signals were developed and stopped by the sequential addition of TMB (3,3',5,5'-tetramethylbenzidine) solution (Sigma #T0440) and 1 N sulfuric acid, and the colorimetric reaction was evaluated by measuring the absorbance at 450 nm. For a competition ELISA, GluA1-Fc was preincubated with 150 μg/ml of soluble LARGE, a competitor, for 30 min before its addition to the LARGE-coated plate. To identify binding preferences of LARGE toward the Fc-GluA1Nt, 2Nt and 4Nt subtypes, different concentrations

of GluA1-family proteins were applied to the LARGE-coated plates as described above. The molarity of each subtype was calculated from the band intensity and molecular weight.

Surface biotinylation

HEK293T cells in 10-cm plates or cultured neurons in 6-well plates were washed with phosphate-buffered saline (PBS) or artificial cerebrospinal fluid (ACSF; 0.15 M NaCl, 10 mM HEPES, 3 mM KCl, 0.2 mM $CaCl_2$ dihydrate, 10 mM glucose), respectively, and placed on ice. The cells were then incubated with 1–1.5 mg/ml sulfo-NHS-SS-biotin (Thermo #21331) for 20 min at 10ºC. Subsequently, the biotin was quenched by incubation with 50 mM glycine for 10 min on ice, followed by washing. The cells were removed by scraping and solubilized in IP buffer containing 1.0% Triton X-100 and 0.1% SDS for 1 h at 4ºC. After solubilization, the cells were centrifuged at 14,000 × g for 15 min, and the pellet was discarded. The supernatant containing biotinylated proteins was incubated with NeutrAvidin-Sepharose beads (Thermo #29200) for 3 h at 4ºC. The beads were then thoroughly washed with IP buffer, and the proteins were eluted for 5 min at room temperature (RT) with protein gel-loading buffer. The total and cell-surface proteins were analyzed by SDS-PAGE, followed by western blot analysis.

Analysis of cell-surface proteins in hippocampal slices using the cross-linking reagent BS^3

Cell-surface proteins were labeled using the membrane-impermeable cross-linking reagent bis(sulfosuccinimidyl) suberate (BS^3, Thermo #21585) as described in previous studies (4), with some modifications. After the cardiac perfusion of mice with cold ACSF, the hippocampi were removed and placed into cold ACSF oxygenated with a 95% O_2/5% CO_2 gas mixture. Each hippocampus was cut into five ~1-mm-thick slices and allowed to float in oxygenated ACSF. Ten slices from two hippocampi were incubated in BS^3 (Thermo-Pierce) solution (2.5 mM BS^3 dissolved in ACSF) for 40 min at 10ºC with gentle shaking. After quenching with ACSF containing 100 mM glycine three times for 5 min each, the slices were processed for solubilization, SDS-PAGE, and western blotting as described above for surface biotinylation.

Preparation of PSD

PSD was prepared as described previously (5), with some modifications.

Western blot analysis

Western blot analyses were performed as described previously (1). The rabbit anti-LARGE antibody (Rb331) (6) was characterized previously. Custom-made rabbit anti-LARGE antibody (UT1002) was generated using a LARGE peptide (KQLSELDEDDLCYEFRRER) through a company service (Open Biosystems, Inc., Huntsville, USA), and used at dilutions of 1:2000. The mouse anti-β-actin (Sigma #A5441) and mouse anti-tubulin antibodies (Sigma #T9026) were used at dilutions of 1:10000 and 1:20000, respectively. For organelle markers, antibodies against rabbit anti-GM130 (Abcam #AB52649), mouse anti-TGN38 (Thermo #MA3-063), and mouse anti-P-cadherin (Abcam #AB22744) were used at dilutions of 1:10000. Mouse anti-GluA1 (Millipore #MAB2263), rabbit anti-GluA2/3 (Millipore #07-598), mouse anti-NR1 (Millipore #05-432), and rabbit anti-NMDAR2B antibodies (Abcam #ab65783) were used at dilutions of 1:1000. Quantitative analyses of band intensities were performed using Image Lab (Bio-Rad).

Immunocytochemistry, microscopy, and image analyses

These processes were performed as described previously (1), with some modifications.

For most immunocytochemistry experiments, cultured neurons were transfected at day in vitro (DIV) 13 and immunostained after 72 hours. For staining, cells were fixed with 4% paraformaldehyde/4% sucrose in PBS for 15 min and permeabilized in 0.2% Triton X-100 for 10 min at room temperature. After blocking with 10% goat serum, the cells were incubated first with primary antibodies, followed by secondary antibodies. Cell surface proteins were immunostained with antibodies prior to permeabilization.

Immunostained cells were imaged using a confocal microscopy system comprising a Nikon A1 microscope with a 60x oil-immersion objective. The images were analyzed using NIS-Element Software (Nikon). Except for one rabbit anti-GluA1-Ct antibody (this paper: JH4294) and rabbit anti-LARGE antibody (this paper: UT1002), the following primary antibodies were used for staining were obtained commercially: mouse, rabbit, or chicken anti-GFP (Neuromabs #75-131, abcam #ab290 or Invitrogen #A10262), GluA1-Nt (Millipore), GM130 (BD Transduction Laboratories or Abcam), and chicken anti-MAP2 (Covance #PCK-554P). All secondary antibodies were purchased from Molecular Probes/Invitrogen/Life Technologies. To quantify the confocal images presented in Fig. 3*C*, the GluA1 intensity in the spine and integrated intensity of individual endogenous GluA1 puncta in the dendritic spine were measured. Images were analyzed using NIS-Elements AR (Nikon). To quantify the confocal images presented in Fig. 1*B*, we focused on the number of overlapping Golgi & GluA1 signal. These co-occurrences were independent of signal intensity. Accordingly, we applied the Manders overlap coefficient, which describes the degree of overlap, to the analysis of co-localization. The images were acquired at a thickness of 0.6 μm. The threshold was determined using a global thresholding process to separate pixels from the background, and the coefficient was calculated from the pixels obtained from all slice images. Customized codes written using C++ and MATLAB were used. To quantify the confocal images presented in Fig. 2 *C* and *E*, fluorescence signals in the soma and dendrites were quantified by measuring the area containing signals above a certain threshold. After normalization, the data were statistically analyzed and plotted as a histogram.

Stereotaxic injection of virus into animals

Adult (9–10 weeks of age) male C57BL/6 mice and Sprague–Dawley rats (body weight: 275–300 g) were assigned to either a control or an experimental group and injected with AAV expressing either scrambled or *LARGE* shRNA, respectively, with enhanced GFP. Specifically, the animals were anesthetized with 2% avertin (Sigma) and placed into a stereotaxic

apparatus (David Kopf instruments), after which the virus solution was injected into the bilateral CA1 region of the hippocampus using the following coordinates: mouse, anteroposterior, -1.95 mm from bregma; mediolateral, ±1.39 mm; dorsoventral -1.66 mm; rat, anteroposterior, -2.4 mm from bregma; mediolateral, ± 2.0 mm; dorsoventral -2.0 mm. For mice, 0.5 µl of virus solution was injected using a *picospritzer* with a glass pipette (diameter, 15–20 µm). For rats, 2 µl of virus solution was injected at a rate of 0.1 µl/min using a syringe pump with a glass pipette (5-µl syringe), and the needle was left in place for at least 10 min post-infusion. The animals were allowed to recover in a heated chamber before waking and were used for behavior tests at least 3 weeks after virus injection. After the tests, the targeting of virus injection into the hippocampal CA1 region was confirmed by the digital imaging of brain slices (2 mm thick) under a blue LED flashlight (DFP-1; NightSea) to excite the GFP. After detecting GFP fluorescence, the fluorescent region of the hippocampus was dissected for biochemical analyses.

Fear conditioning and fear memory tests

Fear conditioning and fear memory tests were performed as previously described (7). The two-pair model of fear conditioning involves placing the animal in the fear conditioning apparatus (Med Associates) for a total of 7 min. Animals were left to explore freely for 3 min. At the 3-min and 5-min time points, an acoustic conditioned stimulus (white noise, 80 dB) was delivered for 30 s, and an unconditioned footshock stimulus was administered through the grid floor during the last 2 s of tone presentation (0.5–0.6 mA for mice, 0.8 for rats) and co-terminated with the tone. Contextual fear memory was evaluated 24 h after paired training by placing the animal into the same training context and measuring freezing behavior for 5 min. The cued fear memory was evaluated at least 4 h after the contextual test by placing the animal in a different context (novel cage floor, lighting, odor, and visual cues) with a 3-min free exploration period. At the 3-min mark, the same acoustic conditioned stimulus was delivered for 3 min, and freezing behavior was measured using Actimetrics FreezeFrame software with

real-time digital video. Data are expressed as the percentage of freezing during each minute or as a mean across all minutes.

Measurement of shock threshold

The shock thresholds for flinching, jumping, and vocalization, which are used as indices of sensitivity to a shock stimulus, were measured as previously described (7). Each animal was placed in the fear conditioning apparatus, and a sequence of single foot shocks was delivered. Initially, a 0.1-mA shock was delivered for 1 s; thereafter, the shock intensity was increased by 0.1 mA at 30-s intervals until an intensity of 1.0 mA was reached. The shock intensity was then decreased by 0.1 mA at 30-s intervals until an intensity of 0.1 mA was reached. Thresholds were then quantified by averaging the shock intensity at which each animal gave a flinching, jumping, and vocalization response.

In vivo field EPSP recordings

Three to 4 weeks prior to *in vivo* field recording, AAV expressing *LARGE* shRNA with GFP was infused into the hippocampal CA1 regions of adult C57BL/6 mice (8–9 weeks old) as described in *Stereotaxic injection of virus into animals*. The stereotaxic unilateral injection of 0.5 µl of higher-titer AAV (1×10^{11} TU/ml) was performed using a stereotaxic, motorized nano-injector (World Precision Instruments) at a rate of 0.1 µl/min via a Hamilton syringe connected to a microinjection pump.

The fEPSPs from the hippocampal CA1 region were recorded as previously described (8). C57BL/6 mice (n = 11–12) were anesthetized with urethane (1.6 g/kg, i.p.; Sigma) and placed into a stereotaxic frame. Rectal temperature was maintained intraoperatively at 36.5ºC ± 0.5ºC using a temperature controller (Harvard Instruments). The scalp was opened and separated. Trephine holes were drilled into the skull, and electrodes were positioned in the area of the hippocampal stratum radiatum. A bipolar stimulating electrode (2.0 mm posterior to bregma, 2.0 mm lateral to midline) was used for Schaffer collateral stimulation,

and a monopolar recording electrode (1.9 mm posterior to bregma, 1.4 mm lateral to midline) was used to record from the CA1 region. The final depths of the electrodes were adjusted to optimize the magnitude of the evoked responses. The fEPSPs were adjusted to 50–60% of the maximal response size for testing. Stimulation was applied using an analog-to-digital interface (1322A; Molecular Devices) and a Digital Stimulus Isolation unit (Getting Instruments). The pyramidal neuron responses to Schaffer collateral stimulation were recorded using a differential amplifier (P55 A. C. pre-amplifier; Grass Instruments) and analyzed using WinLTP software (WinLTP Ltd.). Responses were evoked by single-pulse stimuli delivered at 20-s intervals. A stable baseline was recorded for 30 min. LTP was induced by applying theta-patterned stimulation (TPS, four trains comprising of 10 bursts of five pulses at 400 Hz with a 200-ms inter-burst interval and a 20-s inter-trial interval) to the CA1 and was optimized based on previous studies (8).

Whole-cell patch-clamp recordings for mEPSCs and mIPSC analyses

Cultured hippocampal neurons were prepared as described above. Using a calcium phosphate transfection kit (Invitrogen #K278001), cultured hippocampal neurons were transfected at DIV 9 with cDNA plasmids expressing either scrambled shRNA with enhanced GFP, *LARGE* shRNA with GFP, or *LAEGE* shRNA with GFP and LARGE rescue. The cultured neurons were used for electrophysiological recordings at 3 days' post-transfection. Miniature excitatory postsynaptic currents (mEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) were recorded at room temperature (21–23ºC). Whole-cell voltage-clamp recordings were performed using a multiclamp 700B amplifier (Molecular Devices), filtered at 1 KHz, and digitized at 10 KHz (Digidata 1550; Molecular Devices). Recording pipettes (4–6 MΩ) were filled with the following intracellular solutions, as appropriate: for mEPSC analysis, 140 mM Cs-MeSO4, 8 mM NaCl, 10 mM HEPES, 0.5 mM EGTA, 1 mM $MgCl_2$, 4 mM Mg-ATP, 0.4 mM Na-GTP, 5 mM QX-314; for mIPSC analysis, 130 mM CsCl, 10 mM NaCl, 1.1 mM

EGTA, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM HEPES, 2 mM Mg-ATP. The pH was adjusted to 7.2 using CsOH, with 280–290 mOsm.

Hippocampal neurons on coverslips were transferred to a recording chamber that was continuously perfused with extracellular solution (pH 7.4, 310–320 mOsm) containing 150 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 25 mM glucose. One micromolar tetrodotoxin (Tocris Bioscience #1078), 50 μM DL-AP5 (Tocris Bioscience #3693), and 100 μM picrotoxin (Sigma #P1675) were always included in the extracellular perfusing solution for mEPSC (for mIPSC, 20 μM CNQX (Tocris Bioscience #0190) was used instead of picrotoxin). All recordings were voltage clamped at -70 mV. Acquired data were analyzed using pCLAMP 10.6 (Molecular Devices). Access resistance was continuously monitored. The data were discarded if the R_a varied by >20% during recording. Changes in frequency and amplitude were analyzed, quantified, and presented using traces, cumulative plots, and scatter plots.

Quantification and Statistical Analysis

All statistical analyses were performed using SigmaPlot software (Ver 12; SYSTAT Software). The statistical methods used for particular experiments are noted in the figure legends. Each biological experiment was replicated at least three times using different batches of cells or tissues from different animals. The number of required additional experiments was determined using a power analysis, which was based on a statistical analysis of the data from the first three experiments. The final data sets were analyzed using a two-tailed Student's t-test for experiments with two groups and/or a one (or two)-way ANOVA followed by a *post hoc* Tukey multiple comparison test for experiments with more than two groups. A probability (P) value ≤0.05 was considered significant. All data points were used in plots after confirming a normal distribution. Most values are presented as mean values ± standard errors of the means (SEM). Variations were calculated and are presented as SEMs.

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