Supporting Information

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SI Materials and Methods

Plasmid Construction. pH-GluA2 WT and ΔNSF mutants are cloned into pRK5 vector (BD Biosciences) as previously described (1). C-terminal truncation mutants were made by PCR using forward primer, GAC TCT GGC TCC ACT AAA GAG, which primes position 2014–2034 bp of GluA2, and reverse primers, 856 truncation: ATA GCA AAG CTT GCT AGC CTA GTT AAT ATT CTG TGG ATT CTT TGC, 847 truncation: ATA GCA AAG CTT GCT AGC CTA CTT CAT TCG TTT CGC CTC GGC containing stop codon and external NheI/HindIII site. The resulting PCR products were digested with BspEI (Internal unique GluA2 cutting enzyme) and HindIII and subcloned into pH-GluA2 in pRK5. TeNTLc was generated by PCR by forward primer: AAT CTT GAA TTC GCC ACC ATG CCG ATC ACC ATC AAC AAC (EcoRI site + Kozak sequence + First Met + 5′ end of TeNTLc coding sequence) and reverse primer: AAT CTT AAG CTT TTA AGC GGT ACG GTT GTA CAG (3′ end of TeNTLc coding sequence + Stop codon + HindIII site) using synaptophysin-GFP:IRES:TeNTLc as a template, and subcloned into pRK5 using EcoRI/HindIII site. Construction of the GluA3 plasmids was described previously (2).

Neuronal Culture. Hippocampal neurons from embryonic day 18 (E18) rats were seeded on 25-mm coverslips that were precoated with poly-L-lysine. The cells were plated in Neurobasal media (Gibco) containing 50 U/mL penicillin, 50 mg/mL streptomycin, and 2 mM GlutaMax supplemented with 2% B27(Gibco) and 5% horse serum (HyClone). For TIRF microscopy, we switched neurons to feeding media (plating media without Serum) 24 h after plating and maintained them in serum-free condition thereafter to prevent glial cell growth on the surface of coverslips, which interferes with TIRF microscopy. For surface staining and biotinylation experiments, we switched the media to glia-conditioned NM1 [Neurobasal media with 2 mM GlutaMax, 1% FBS, 2% B27, 1 × FDU (5 μM uridine (F0503; Sigma), 5 μM 5-Fluor-2'-deoxyuridine(U3003; Sigma)] at DIV6 and maintained thereafter. In both conditions, neurons were subsequently fed twice a week by changing half volume of the feeding media.

Visualization of Receptor Insertion by Total Internal Reflection Microscopy. The TIRF microscopy imaging system was based on a Zeiss AxioObserver microscope (Carl Zeiss Microimaging) (3). The excitation laser was a Coherent Sapphire 488 nm—50 mW (Coherent). The laser was coupled to a Zeiss TIRF slider via a KineFLEX-P-2-S-488–640-0.7-FCP-P2 fiber optics (Point Source). A Z488RDC dichroic mirror (Chroma Technology) was used to reflect the incoming laser onto a Zeiss a plan 100× objective (N.A. = 1.45, Carl Zeiss). An ET525/50 emission filter was used for pHluorin fluorescence detection (Chroma Technology). An EMCCD camera (ImagEM C9100-13; Hamamatsu Photonics) was used as detector. To detect dim signals, the EMCCD gain was set to maximal. The camera was maintained at −85 °C during the experiment using a JULABO HF25-ED heating and refrigerated circulator (JD Instruments). An Unblitz LS6ZM2 shutter controlled by VCM-D1 (Vincent Associates) was integrated between the laser head and the fiber launcher to control the laser. Data were acquired using Zeiss AxioVision software (Carl Zeiss) or SlideBook (Intelligent Imaging Innovations). Twenty-four to 36 h before the experiment, hippocampal neurons were transfected with pHluorin-tagged AMPA receptors using LipofectAMINE2000 in serum and antibiotic free conditions for 1 h. Neurons between ages of 18–19 DIV were used for imaging experiments unless otherwise specified. All of the imaging experiments were carried out in E4 solutions (124 mM NaCl, 3 mM KCl, 10 mM D-(+)-Glucose, 2.5 mM $CaCl_2$, 1.25 mM $MgCl_2$, 15 mM Hepes, pH 7.4–7.45) at 35 °C using stage heater. Live cell images were captured every 5 sec with exposure time 200 msec for 5–10 min (60–120 frames) to generate each movie. We also imaged GluA2 insertion at higher frame rates (4 frames/sec), but to overcome the low insertion frequency of GluA2, lower frame rates and longer recording times were preferred, because this avoided unnecessary photo-bleaching and increased the number of insertion events detected. To increase the signal-noise ratio, we typically performed 1 min photobleach of preexisting surface AMPARs before data acquisition. Recordings were analyzed using ImageJ and insertion events lasting over two frames (10 sec) were registered as events manually. Y–t rendering images were generated by rotating the original xyt stack 90° along *y* axis using maximum intensity projection algorithm. Excitatory neuronal activity was acutely suppressed by applying a mixture of tetrodotoxin (TTX, 1 μM), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f] quinoxaline-2,3-dione (NBQX, 20 μM) and DL(−)-2-amino-5-phosphonovaleric acid (AP5, 200 μM) for 15 min before data acquisition. Endosomal trafficking was blocked by preincubated with TAT-Syn7ΔTM or TAT-Syn13ΔTM (3.5 μM) for 1 h at 37 °C and maintained perfusion throughout the recording.

Sindbis Virus Infection and Surface Biotinylation. pH-GluA2 WT, ΔNSF, R607Q, and R607QΔNSF in pRK5 were digested with XhoI/ HindIII restriction enzymes and blunt ends were generated by Klenow fragment (New England BioLabs). Sindbis backbone vector, pSinRep5(nsp2s) were digested with StuI and pH-GluA2 inserts were subcloned into this vector. Sindbis virus production was performed according to Invitrogen manual. In brief, after linearization of pH-GluA2 in pSinRep5(nsp2s) by NotI and DH(26S)5′tRNA by XhoI, in vitro transcription was performed using mMESSAGE-mMACHINE Sp6 kit (AM1340; Ambion). Both in vitro transcripted RNA were immediately electroporated into BHK cells using GenePulser (BioRad). Forty-eight hours after electroporation, conditioned media were collected and centrifuged at 25,000 rpm for 2 h to concentrate virus. Concentrated virus was dissolved into 100 μl Neurobasal media. A titration assay was performed by infecting virus into hippocampal neurons and counting pHluorin signal positive neurons. We used the virus at the concentration where 90–100% neurons in a dish are infected based on previous titration assays. Typically, DIV18 hippocampal neurons were infected by 1 h and medium was replaced with saved conditioned media for another 24 h. After incubation, cells were washed with E4 twice, cell surface protein were biotinylated with 1 mg/mL sulfo-NHS-SS-biotin for 20 min on ice. The remaining biotin was quenched by washing the cells with ice-cold TBS containing 50 mM glycine for 5 min twice. Immediately after quenching, neurons were lysed with modified RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA) supplemented with complete protease inhibitor mix (Roche). Biotinylated cell surface proteins were precipitated using neutral-avidin agarose (Pierce). Precipitated cell surface proteins and total cell lysates were separated by 7.5% SDS/PAGE and blotted with anti-GFP antibody (JH4030: rabbit-polyclonal, respectively). Specific isolation of cell surface proteins was confirmed by blotting the same membranes with anti-tubulin antibody (Sigma).

Newly Inserted Receptor Detection by Thrombin-Cleavage Method. Newly inserted AMPAR assay were performed as described previously (4). In brief, 18–19 DIV hippocampal neurons transfected with each pH-T-GluA2 constructs. Transfected neurons were treated for 5 min at room temperature with thrombin (1 unit/mL in DMEM; GE Healthcare). After thorough washing with DMEM, neurons were returned to 37 °C for various times to allow for surface insertion of new receptors. Next, neurons were surface-labeled with rabbit polyclonal anti-GFP antibody (1 mg/mL, JH4030) for 30 min at 4 °C to visualize the surface pH-T-GluA2, then washed with ice-cold DMEM and fixed for 10 min in 4% paraformaldehyde/ 4% sucrose in PBS. After three washes with PBS, neurons were incubated with Alexa546 conjugated anti-rabbit IgG (Molecular Probes) for 1 h at room temperature. Images were acquired with Carl Zeiss LSM510 confocal microscope. Steady state surface GluA2 was detected using the same method except without thrombin-cleavage. The ratio of newly inserted/ steady state receptors was calculated for each time point. We determined steady state GluA2 as 1.00.

Statistics. All of the statistical tests were performed using Excel (Microsoft) or SPSS software 9.0 (SPSS). Values were expressed as mean ± SEM unless otherwise specified. Comparisons for two groups of data were done by two-tailed student's *t* test. Multiple comparisons were done by one-way ANOVA followed by Tukey posthoc test. ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

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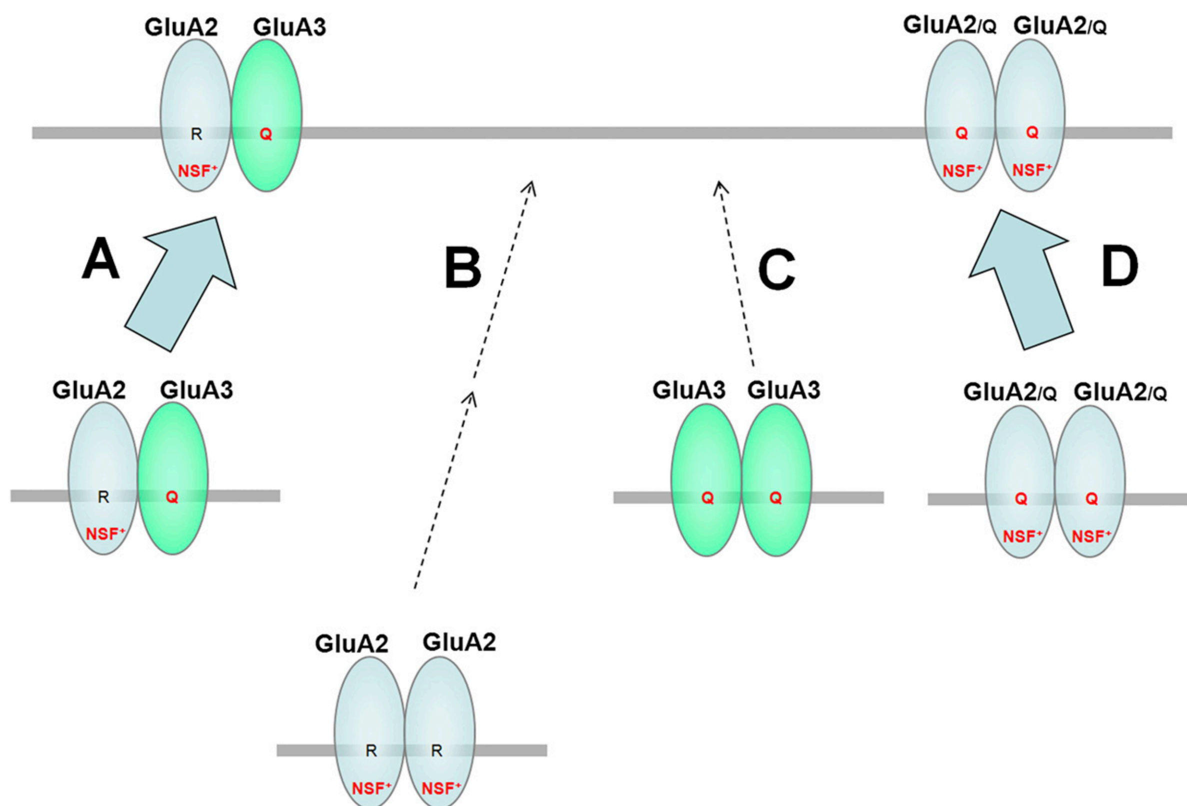

Fig. S1. Schematic diagram for efficient insertion of GluA2/3 onto plasma membrane. (*A*) GluA2 forms a complex with GluA3. Under this condition, GluA2 provides the NSF binding sequence and GluA3 provides the unedited residue (Q) that is required for exit from ER resulting in efficient GluA2/3 insertion into plasma membrane. (*B*) GluA2 forms a complex with GluA2. Under this condition, GluA2 provides the NSF binding sequence, but not the unedited residue (Q) in pore region, which results in ER retention of these receptors. (*C*) GluA3 forms a complex with GluA3. Under this condition, GluA3 provides the unedited residue (Q) residue but lacks the NSF binding sequence, thus these homomeric receptors cannot be efficiently inserted onto plasma membrane. (*D*) Unedited GluA2 forms complex with unedited GluA2. Under this condition, unedited GluA2 provides both the NSF binding sequence and the unedited residue (Q), and can be efficiently inserted onto plasma membrane. However, most of GluA2 is edited in the adult brain.

Movie S1. Real time visualization of GluA2 insertion events (in comparison with GluA1) (*Part 1*) Visualization of GluA2 insertion events. pHluorin-tagged GluA2 was transfected in hippocampal neurons and insertion events were observed at DIV19 by TIRF microscopy. (*Part 2*) Visualization of GluA1 insertion events—pHluorin-tagged GluA1 was transfected in hippocampal neurons and insertion events were observed at DIV19 by TIRF microscopy. Note that there were more frequent and brighter insertion events in case of GluA1 compared with GluA2 when imaged under identical conditions (i.e., exposure time, laser power, CCD camera gain, and speed). Time [h:min:sec.millisecond] is indicated at upper left side. (Scale bar, 10 μm.) (*Part 3*) Visualization of an individual GluA2 insertion event. pHluorin-tagged GluA2 was transfected into hippocampal neurons and was observed at DIV19 by TIRF microscopy.

Movie S1