Plasma membrane insertion of the AMPA receptor GluA2 subunit is regulated by NSF binding and Q/R editing of the ion pore

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Abstract

GluA2 is the major excitatory neurotransmitter in the mammalian CNS. Ionotropic glutamate receptors are classified into several groups: AMPA, kainate, and NMDA receptors. AMPA receptors (AMPARs) are ligand-gated cation channels that mediate the majority of the fast excitatory synaptic transmission (1), whereas NMDA receptors (NMDARs) are critical for the induction of specific forms of synaptic plasticity by dynamically regulating synaptic expression of AMPARs (2–4).

The AMPARs consist of four subunits; GluA1, -2, -3, and -4 (5, 6). Several studies have shown that GluA2 interacts with PDZ domain containing proteins such as GRIP1/2 and PICK1 via its PDZ ligand at its C terminus. GRIP1/2 stabilizes surface expression of GluA2 or promotes receptor recycling to the plasma membrane (7, 8), whereas PICK1 promotes GluA2 endocytosis or inhibits receptor recycling to the plasma membrane (9–12). Disrupting GluA2–PICK1 interactions blocks the expression of both hippocampal and cerebellar LTD (11, 12). In contrast, N-ethylmaleimide-sensitive factor (NSF), an essential component of SNARE-mediated membrane fusion machinery (13–15), binds to the GluA2 juxta membrane region (16–19). Disruption of NSF binding to GluA2 by a peptide inhibitor decreases both AMPAR mediated synaptic transmission (16, 17) and GluA2 surface expression by disassembling GluA2–PICK1 complexes (20), whereas overexpression of NSF increases surface expression of GluA2 (21). However, the precise regulatory mechanisms underlying GluA2 delivery to the surface plasma membrane remain elusive, mainly because exocytosis and endocytosis of AMPAR containing vesicles are highly dynamic and have not been kinetically resolved. Most conventional assays for monitoring AMPARs surface expression, including surface biotinylation or surface staining using antibodies against extracellular domains or extracellular tags, lack sufficient temporal resolution to isolate the kinetics of insertion. Therefore, direct visualization of plasma membrane insertion GluA2 containing receptors is a prerequisite to separate exocytotic events from rapid endocytosis of the receptors. Here, we visualize plasma membrane insertion of GluA2 containing vesicles by imaging supercceptive pHluorin-tagged GluA2 in neurons under total internal reflection fluorescence (TIRF) microscopy. Using this approach, we visualize individual insertion events of GluA2 containing vesicles. These events are blocked by tetanus toxin light chain indicating that they are mediated by SNARE machinery containing synaptobrevin (VAMP-2). We further demonstrated that approximately 50% of newly inserted GluA2 originate from recycling endosomes. We find that both the RNA editing of the Q/R site in the pore region of GluA2/3 and the NSF binding site of GluA2 strongly regulate surface delivery of AMPARs. These results describe the regulation of the insertion of GluA2 into the plasma membrane by distinct structural elements of the GluA2 subunit critical for the synaptic delivery of GluA2 containing AMPA receptors.

Results

Direct Visualization of GluA2 Insertion Events. To directly visualize GluA2 plasma membrane insertion in hippocampal neurons, we used total internal reflection microscopy (TIRFM) to image the fluorescence signal of supercceptive pHluorin-tagged GluA2 (pH-GluA2) near the cell surface. TIRFM allows excitation light to reach approximately 100 nm from the cover glass surface, enabling tracking of pHluorin-tagged receptor insertion at the plasma membrane. Moreover, the fluorescence of the pHluorin tag is quenched in the lumen of intracellular acidic organelles, including endosomal and Golgi compartments and is only detectable when these vesicles fuse with the plasma membrane and are exposed to the neutral pH of the extracellular environment (22). Thus, by imaging pH-GluA2 under TIRFM, one can visualize newly inserted cell surface pHluorin-tagged AMPA receptors (23, 24). Using TIRF imaging pH-GluA2 signals were detected distributed on the cell body and dendrites at steady state. After complete bleaching of the preexisting pH-GluA2 signal on the cell surface to increase the signal to noise ratio, we were able to visualize many individual insertion events over a 10-min recording period (Fig. L4A). An example of an image sequence of GluA2 insertion is shown in Fig. L4B. In the image sequence shown the cells were imaged every 5 sec (Movie S1). As shown in Fig. L4B GluA2 fluorescence appeared and laterally diffused from the point of insertion within the plasma membrane. The average signal intensity at the center of the insertion point (blue: diameter 1 μm), the medium proximal region surrounding the insertion point (pink: 4 μm), and the distal region (green: 8 μm) is shown in Fig. L4A demonstrating that the newly inserted GluA2 rapidly diffused radially from the point of insertion. Y–t rendering images were generated by rotating the original x–y–t stack 90° along the...
y axis, and the maximum intensity of each x line was projected onto a single pixel of the y axis using a maximum intensity projection (MIP) algorithm (Fig. 1A) to show the frequency and time course of the insertion events.

We verified these as true GluA2 receptors insertion events by coexpressing tetanus toxin light chain (TeNTLc) (25) (Fig. 1B). TeNTLc cleaves synaptobrevin/VAMP2, an essential component of the SNARE complex, and thus inhibits synaptobrevin/VAMP2 mediated exocytosis. TeNTLc abolished both GluA1 and GluA2 insertion events. Examples of the MIPs and quantitation of the insertion events are shown (means ± SEM, n = 12).

Fig. 1. Direct imaging of GluA2 plasma membrane insertion events. (A) Representative images of GluA2 insertion events over a 10-min time period in hippocampal neurons visualized using TIRF microscopy (Scale bar, 10 μm.) (2). Representative images of the time course of pH-GluA2 insertion and diffusion (3). Quantification of fluorescence change over time demonstrates lateral diffusion of pH-GluA2 following insertion (4). Y-t projection image shows MIP of the insertion event shown in (2) and (3). (B) Insertion of GluA1 and GluA2 is dependent of VAMP2. Cotransfection of TeNTLc abolished both GluA1 and GluA2 insertion events. Examples of the MIPs and quantitation of the insertion events are shown (means ± SEM, n = 12). (C) Effect of recycling inhibitor (TAT-Syn13ΔTM) on GluA1 and 2 insertion events frequency per 10 min. Only TAT-Syn13ΔTM reduced both GluA1 and 2 insertion. Examples of the MIPs and quantitation of the insertion events are shown (means ± SEM, n = 10). (D) Acute activity block (TTX/CNQX/APV treatment) abolished most of GluA1 insertion, whereas this treatment had a smaller effect on GluA2 insertion. Examples of the MIPs and quantitation of the insertion events are shown (means ± SEM, n = 11).

AMPARs required for LTP expression are thought to originate from recycling endosomes (27). Perfusion of TAT-Syn7ΔTM peptide, which blocks transport of recycling endosomes to the plasma membrane, inhibited approximately 50% of both GluA1 and 2 insertion, whereas perfusion of TAT-Syn13ΔTM, a blocker of transport from early endosomes to late endosomes did not affect either GluA1 or 2 exocytosis (Fig. 1C). These results in-
AMPAR membrane traffic of AMPAR subunits bind many proteins that regulate basal GluA2 insertion, we next used point mutations (V848L/A849T/P852T) that specifically eliminate NSF binding to GluA2, whereas the R607Q mutation facilitated surface expression. GluA2 containing both mutations (R607Q/ΔNSF) also showed reduced surface expression of GluA2 compared with R607Q, again indicating that the NSF binding site affects surface expression levels of edited and unedited GluA2.

As a complementary method to determine requirements for GluA2 surface delivery in neurons, we modified pH-GluA2 to insert a thrombin cleavage sequence (LVPRGS) between the pHluorin-tag and GluA2 sequence (designated as pH-T-GluA2). Surface delivery of pH-T-GluA2 can be monitored using a thrombin cleavage assay (31, 32) to study the kinetics of AMPA receptor surface delivery (Fig. 3B). Thrombin pretreatment cleaves preexisting surface pH-T-GluA2 receptors to allow specific detection of subsequently inserted receptors. After thoroughly washing the coverslip, cells were placed into conditioned media, indicating that the NSF binding site affects surface expression levels of edited and unedited GluA2.

Fig. 2. The NSF binding site is important for GluA2 insertion. (A) Mapping of GluA2 C-terminal region responsible for efficient insertion. GluA2 C-terminal sequence; the truncation and point mutants used in this study are indicated. (B) The GluA2 856t mutant has no effect on GluA2 insertion, whereas GluA2 847t mutant abolished its insertion. Examples of the MIPs and quantitation of the insertion events are shown (means ± SEM, n = 12). (C) Effect of GluA2 ΔNSF and R607Q to GluA2 insertion frequency. Mutation of the NSF site and the Q/R site significantly affect the insertion frequency. Examples of the MIPs and quantitation of the insertion events are shown (means ± SEM, n = 14).
ΔNSF GluA2 mutant showed reduced kinetics of surface delivery of GluA2 both in edited and unedited (R607Q) forms. These results indicate that the NSF binding sequence is indispensable for efficient trafficking of GluA2 to the cell surface.

Presence of the NSF Binding Site and an Unedited Q/R Site Is Required for Efficient Insertion of GluA2/3 Heteromers.

To visualize GluA3 insertion, pHluorin-tagged GluA3 subunits were transfected into hippocampal neurons and imaged by TIRF microscopy. As shown in Fig. 4A, compared with GluA1, both GluA2 and 3 have lower insertion frequencies (GluA1 87.5 ± 7.7 events/10 min; GluA2 14.6 ± 3.2 events/10 min; GluA3 14.1 ± 5.8 events/10 min). We used the edited version (R) of GluA2 (pH-GluA2/R) for comparison because the majority of GluA2 in the mature brain is edited (R) (designated as GluA2/R), whereas GluA1 and 3 are unedited (Q) (designated as GluA1/Q, GluA3/Q, respectively). To further investigate the mechanisms of GluA2/3 exocytosis, we coexpressed pH-GluA2/R with various GluA3 constructs, and examined pH-GluA2/R exocytosis (Fig. 4B). Interestingly, coexpression of GluA3ΔNSF greatly facilitates GluA2 exocytosis (pH-GluA2ΔNSF+Vector: 13.0 ± 2.7 events/10 min, pH-GluA2+GluA3ΔNSF: 130.1 ± 14.1 events/10 min: **P < 0.01), whereas coexpression of pH-GluA2/R with a construct containing an artificial edited mutation in GluA3 pore region (Q612R) had no effect (pH-GluA2ΔNSF+R3 Q612R: 16.0 ± 6.6 events/10 min: P = 0.58 compared with pH-GluA2ΔNSF + mock vector). This result suggests that the presence of unedited residue (Q) in an AMPAR complex is critical for receptor surface delivery. Deletion of the complete C-terminal region of GluA3 (GluA3ΔC) did not inhibit the enhancing effect of GluA3ΔNSF on pH-GluA2ΔNSF insertions [pH-GluA2ΔNSF+GluA3ΔC: 116.0 ± 19.8 events/10 min (P = 0.10) compared with pH-GluA2ΔNSF + GluA3ΔQ], indicating that GluA3 C-terminal sequence is not critical for the ability of GluA3ΔNSF to facilitate GluA2 exocytosis. These results indicate that facilitation of edited GluA2 insertion by GluA3 depends on the unedited residue (Q) of GluA3.

We examined the importance of NSF binding sequence for heteromeric receptor (GluA2/3) surface delivery (Fig. 4C). Coexpressing GluA3ΔQ with pH-GluA2ΔNSF had no effect in GluA2 exocytosis (pH-GluA2ΔNSF+Vector: 4.3 ± 1.7 events/10 min: pH-GluA2ΔNSF+GluA3ΔQ: 7.5 ± 1.9 events/10 min: P = 0.26). To test whether the NSF binding site had to be present in the GluA2 subunit we generated GluA3ΔQ mutants that can artificially interact with NSF (GluA3ΔQ NSF+ by mutating GluA3 to contain a NSF binding site identical to GluA2 (L853V/T854A/T857P) (29). Coexpressing GluA3ΔQ NSF+ with pH-GluA2ΔNSF could rescue the GluA2ΔNSF insertion deficiency (pH-GluA2ΔNSF+GluA3ΔQ NSF+: 101.5 ± 9.5 events/10 min: **P < 0.01).

Fig. 3. The NSF binding site is important for efficient delivery of GluA2 to plasma membrane. (A) Surface expression of GluA2 constructs in hippocampal neurons probed using a surface biotinylation assay. Hippocampal neurons were infected with Sindbis virus expressing the indicated GluA2 construct and the surface receptor analyzed using biotinylation techniques. The surface fraction precipitated by streptavidin-beads (surface) and the total lysate (total) is shown. The graph shows the ratio of surface GluA2/total GluA2 (means ± SEM, n = 3). (B) Newly inserted GluA2 time course. Neurons expressing pH-GluA2 were treated by Thrombin for 5 min. After thoroughly washing the coverslip, the cells were incubated for the indicated times. The graph shows the recovery of surface receptors over time (means ± SEM, n = 3, each time point). We set steady state ratio of surface/total GluA2 is 1.
P < 0.001 compared with pH-GluA2/RΔNSF + vector), indicating that both the NSF binding sequence and an unedited residue (Q) is required for efficient insertion of GluA2/3 heteromers. Examples of the MIPs and quantitation of the insertion events are shown (means ± SEM, n = 11). (C) Effect of coexpression of GluA3ΔQ containing an artificial NSF binding sequence on GluA2ΔNSF insertion frequency. Examples of the MIPs and quantitation of the insertion events are shown (means ± SEM, n = 12). (D) Effect of Glur2 coexpression for pH-GluA3ΔQ insertion events. GluA2 carrying the NSF binding sequence facilitates GluA3 insertion event frequency. Examples of the MIPs and quantitation of the insertion events are shown (means ± SEM, n = 12).

**Discussion**

We have directly visualized the plasma membrane insertion of GluA2/3 containing intracellular vesicles by imaging superecliptic pHluorin-tagged AMPARs using TIRFM. We have shown (i) GluA2 plasma membrane insertion events contain only a few receptors compared with that of GluA1; (ii) GluA2 insertion events can be blocked by tetanus toxin light chain, indicating that they are mediated by synaptobrevin/VAMP-2 containing SNARE machinery; (iii) approximately 50% of GluA1 and 2 insertion events originate from recycling endosomes; (iv) GluA1 exocytosis is mostly (approximately 88%) activity dependent, whereas GluA2 exocytosis is largely (approximately 70%) constitutive, but approximately 30% of GluA2 exocytosis remains activity-dependent; (v) Both the NSF binding sequence in the GluA2 C terminus and an unedited residue (Q) in the pore region facilitate GluA2 receptor surface insertion; (vi) and GluA2/3 heteromeric receptors behave similarly to GluA2 homomers and the insertion of GluA2/3 heteromers requires the NSF binding site in GluA2 and the unedited Q/R site in GluA3.

Previous studies have shown that the editing of the Q/R residue in the pore region of GluA2 plays an important role in the assembly of GluA2 subunits and the exit of GluA2 subunits from the ER (30). Overexpression of the GluA2ΔQ subunits results in ER retention of the GluA2 subunit, whereas overexpressed GluA2ΔQ subunits are not retained in the ER. Interestingly, our data demonstrates that editing of this site dramatically decreases the rate of the direct insertion of GluA2 into the plasma membrane analyzed by TIRFM and pH-GluA2. This may be due to an increase in the pool of GluA2ΔQ in intracellular vesicles able to fuse with the plasma membrane because of the lack of retention of the GluA2ΔQ subunit in the ER. Alternatively, editing the Q/R site may have a direct independent effect on the insertion of the GluA2 subunit into the plasma membrane.

Disrupting the interaction between GluA2 and NSF results in the rundown of AMPA responses in neurons, suggesting that NSF is important for incorporation and maintenance of AMPARs at synapses (16, 17, 29). Recent studies have suggested that NSF may...
regulate the delivery and/or lateral mobility of GluA2 containing receptors from extrasynaptic sites to synapses. Our results clearly show that the direct plasma membrane insertion of GluA2 is also regulated by NSF binding.

A schematic model of GluA2/3 receptor surface expression requirements is shown in Fig. S1. In the brain, most GluA2 is edited at the Q/R site in the ion channel pore (33, 34). Thus, heteromerization of GluA2/R with GluA3 cannot be detected, possibly because it is not inserted into the synaptic membrane surface (35). Similarly, overexpressed GluA3 in cerebellar Purkinje cells is not incorporated into synapses unless mutated to contain an NSF binding site (29). Interestingly, unedited GluA3 homomeric receptors are calcium permeable, which could lead to excitotoxicity and may cause neuronal cell death. The low efficiency of surface delivery of calcium permeable (unedited) GluA3 may provide an intrinsic neuroprotective mechanism to prevent the insertion of high levels of calcium permeable AMPAs.

The results presented here reveal insights into the regulation of GluA2 plasma membrane insertion under basal conditions. It will be interesting to use similar methods to investigate whether the same or additional molecular mechanisms control AMPAR insertion during forms of synaptic plasticity such as LTP.
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 CTG TGC, 847 truncation: ATA GCA AAG CTT GCT AGC CTA CTA TCG TTT CTC GTC GCC 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. pENTLE 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 pENTLE coding sequence) and reverse primer: AAT CTT GAA TTC GCC ACC ATG CCG ATC ACC ATC AAC AAC (EcoRI site + Kozak sequence + First Met + 5′ end of pENTLE coding sequence + Stop codon + HindIII site) to generate 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 Neurobasal media with 2 mM GlutaMax, 1% FBS, 2% B27, 1×FDU (5 μM uridine (F0503; Sigma), 5 μM 5-flour-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 aplan 100x 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 (Δμfl/M uridine (F0503; Sigma), 5 μM 5-flour-2′-deoxyuridine (U3003; Sigma)) was used. The results were expressed in percent. All the experiments were carried out in 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-benzof[l] 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 Stul 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 transcribed RNA were immediately electroporated into BHK cells using GenePulsor (BioRad). Forty-eight hours after electroporation, concentrated 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 (JH4306; rabbit-polyclonal, respectively). Specific isolation of cell surface proteins was confirmed by blotting the same membranes with anti-tubulin antibody (Sigma).
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).

1. Lin DT, Huganir RL (2007) PICK1 and phosphorylation of the glutamate receptor 2 (GluR2) AMPA receptor subunit regulates GluR2 recycling after NMDA receptor-induced internalization. J Neurosci 27:13903–13908.

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.