Supplementary Figure 1: Acute knockdown of endogenous PSD-95 by shRNA. (a) Control cell infected with a lentivirus expressing GFP shows the typical punctuate pattern of staining for endogenous PSD-95. Cell expressing sh95 + GFP shows almost complete disappearance of endogenous PSD-95. The boxed regions in the two PSD-95 panels are shown magnified in the lowest panels. Scale bar, 20 μm. (b) Quantitation of PSD-95 levels in control and sh95 infected cells shows that sh95 is highly effective in knocking down endogenous PSD-95. *** indicates p < 0.001.
**Supplementary Figure 2:** Example of dual receptor labeling assay showing that the second, secondary antibody used to label internalized AMPARs does not label surface AMPARs. The two upper panels show that in an unpermeabilized cell, application of a saturating concentration of the first, Alexa-568 conjugated secondary antibody that is used to stain surface GluR1 (upper left panel) prevented any further detectable staining of surface GluR1 upon subsequent application of the second, Cy5 conjugated, secondary antibody (upper right panel). The lower left panel shows surface GluR1 staining following application of the saturating concentration of the first Alexa-568 conjugated secondary antibody prior to permeabilization of the cell. The lower right panel shows GluR1 staining following permeabilization of the cell with 0.1% Triton X-100 for 30 min at room temperature and application of the second, Cy5 conjugated secondary antibody that now labels internalized GluR1-containing AMPARs. These experiments show that the second, Cy5 conjugated secondary antibody that was used for labeling internalized AMPARs does not label surface AMPARs, indicating that the saturating concentration of the first, Alexa-568 conjugated secondary antibody occupied all surface AMPARs. Scale bar, 20 µm.
Supplementary Figure 3: Knockdown of PSD-95 does not affect the synaptic localization of NMDARs. Representative images (a) and quantitation (b) of NMDAR synaptic localization determined by calculating the percentage of synapses (defined by Bassoon puncta) that co-localized with detectable levels of NR1. Scale bar, 10 μm. n.s indicates p > 0.05.
Supplementary Information

Detailed Methods

**Lentivirus and construct preparation.** To generate lentiviruses, the expression vector, the HIV-1 packaging vector Δ8.9, and the VSVG envelope glycoprotein vector were cotransfected into HEK293 cells using FUGENE6 transfection reagent (Roche). The expression vector that we used in this study is called FUGW and it is a dual promoter lentiviral vector in which the human H1 promoter drove expression of sh95 and an ubiquitin promoter simultaneously drove expression of GFP or a GFP-fusion protein\(^1\). 48 hr after transfection, supernatants of culture media were collected. To clone mouse AKAP150 cDNA, total RNA from adult mouse brain hippocampi was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s instruction. AKAP150 cDNA was generated by reverse transcription using Superscript reverse transcriptase (Invitrogen). AKAP150 cDNA was further amplified using Pfx-DNA polymerase (Invitrogen) and cloned into the BamHI site of the FUGW vector so that it was C-terminally tagged with GFP for lentiviral expression. The AKAP\(\Delta\)PP2B construct was generated by deleting the reported PP2B binding domain of AKAP150 (aa 613-655)\(^3\,^4\). High overexpression of AKAP150 or AKAP\(\Delta\)PP2B in neuronal cultures was deleterious to cell health and thus low titers of virus were used in the experiments illustrated in Figure 7. For biochemical studies in HEK293 cells, mouse AKAP150 was cloned into pCMV-HA vector (Clontech) so that it was tagged with an HA-epitope at its N-terminus.

**Immunocytochemistry and image analysis**

To assay total AMPAR endocytosis (Figure 1), surface AMPARs were labeled in live neurons by 15 min incubation at 37°C with a rabbit polyclonal antibody (Calbiochem) directed against the N-terminus of the GluR1 subunit (1:20 in conditioned media, i.e. MEM + 0.5 mM glutamine). After washout of the antibody cells were treated with appropriate mixtures of antagonists (1 \(\mu\)M TTX + 100 \(\mu\)M LY341495 + 20 \(\mu\)M DNQX for NMDA application; 1 \(\mu\)M TTX + 100 \(\mu\)M LY341495 + 50 \(\mu\)M APV for AMPA application; 1 \(\mu\)M TTX, 20 \(\mu\)M DNQX and 50 \(\mu\)M APV for DHPG application; all drugs from Tocris) for 5 min in conditioned media. Subsequently, NMDA (100 \(\mu\)M), AMPA (100 \(\mu\)M) or DHPG (50 \(\mu\)M) (all from Tocris) were applied for 3-5
min in the presence of appropriate antagonists. The agonist was then washed out and cells were further incubated in the presence of antagonists. All experiments were allowed to incubate for a total of 15 min at 37°C after initial agonist application. Cells were next chilled on ice and antibodies remaining on surface receptors were stripped with an acidic solution (0.5 M NaCl, 0.2 N acetic acid) for 3 min on ice as previously described\(^5\). Cells were then fixed in 4% PFA, permeabilized in 0.1% Triton X-100 for 30 min at room temperature and stained with goat anti rabbit Alexa-568 secondary antibody (Molecular probes, Invitrogen).

To measure both internalized and remaining surface AMPARs (Figures 2-7) after agonist application, surface receptors were labeled with the rabbit polyclonal antibody directed against the N-terminus of the GluR1 subunit in live cells and agonists were applied in the presence of the appropriate antagonists as just described. After the 15 min incubation period, cells were fixed in 4% PFA for 15 min on ice without permeabilization and surface receptors were visualized by using saturating amount of goat-anti rabbit Alexa 568 conjugated secondary antibody (Molecular Probes, Invitrogen), followed by permeabilization of cells with 0.1% Triton X-100 for 30 min at room temperature and staining of internalized receptors with donkey-anti rabbit Cy5-conjugated secondary antibody (Jackson Immunoresearch). As previously reported\(^6\), control experiments indicated that application of the first secondary antibody was effective in staining all remaining surface receptors as application of the second secondary antibody yielded no detectable Cy5 signal in non-permeabilized cells (Supplementary Fig. 2). We used a Cy5 conjugated secondary antibody to label the internalized receptors since Cy5 is not detectable in the visible range and thus allowed us to image cells in every condition in a blinded fashion. eGFP fluorescence was visualized by using anti-GFP monoclonal antibody (Chemicon) and goat-anti mouse Alexa 488-conjugated secondary antibody (Molecular probes, Invitrogen). Basal endocytosis of AMPARs was assayed using the same protocol in cells not treated with any agonist.

All analyses were done blind using raw images. Untreated and treated cells from the same culture preparation were always compared with one another. Images from each experiment were thresholded using identical values for different experimental conditions and the total thresholded area of fluorescently labeled surface and internalized receptors was measured using Metamorph. To measure the surface receptors in all our assays, surface fluorescence was divided by cell area, which was determined by measuring background fluorescence using a low
threshold level. These values were then normalized to the average surface fluorescence of untreated control cells. For figure 1, total AMPAR endocytosis was calculated using the total internalized fluorescence measurements for each cell normalized to untreated control cells. For calculation of the proportion of surface AMPARs that were endocytosed (Figures 2-7), intracellular fluorescence was divided by total fluorescence (intracellular plus surface) for each cell. These values were then normalized to those of untreated control cells from the same experiment. Each experimental treatment and analysis was performed on a minimum of two coverslips with most experiments using on average six to eight coverslips. For presentation, images were processed using Adobe Photoshop software (Adobe Systems) by adjusting brightness and contrast levels to the same degree for all conditions illustrated in each experiment.

Synaptic GluR1 puncta were quantified by colocalization of surface GluR1 puncta and presynaptic Bassoon puncta. After live cell staining with the anti GluR1 N-terminus rabbit polyclonal antibody cells were either not treated (control) or agonists (NMDA or AMPA) were applied as described above. Cells were then fixed in 4% PFA on ice for 15 min without permeabilization and were incubated with goat-anti rabbit Alexa-568 secondary antibody for 1 hr at 37°C. Cells were then permeabilized in 0.1% Triton X-100 for 30 min at room temperature and subsequently were incubated with a monoclonal antibody against Bassoon (Stressgen) for overnight at 4°C. Subsequently, cells were incubated with goat-anti mouse Alexa-488 secondary antibody for Bassoon puncta visualization. To determine the efficiency of sh95 in reducing endogenous PSD-95 levels, cells were infected with the sh95-containing lentivirus and 5-7 days after infection were fixed with 4% PFA, permeabilized using 0.1% Triton X-100 and stained for PSD-95 with a monoclonal anti-PSD-95 antibody (Sigma) and goat-anti mouse Alexa-568 secondary antibody.

The effect of sh95 on the synaptic localization of NMDARs was studied by quantification of colocalization of NR1 puncta and the presynaptic Bassoon puncta in control and sh95 treated cells. Briefly, cells were first infected with sh95 and 5-7 days after infection cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 30 min at room temperature. NMDARs were then detected using a monoclonal antibody to the intracellular loop between transmembrane regions III and IV of the NR1 subunit (PharMingen) and synapses were simultaneously labeled using rabbit anti-Bassoon polyclonal antibody (Synaptic Systems).
To study the effect of NMDA application on the synaptic localization of PSD-95 and AKAP150, cells were infected with various PSD-95 and AKAP150 constructs. 5-7 days after infection cells were treated with NMDA using identical protocols as described above. Cells were then fixed in 4% paraformaldehyde and permeabilized using 0.1% Triton X-100 as described earlier. Subsequently, endogenous PSD-95 was stained with monoclonal anti-PSD-95 antibody (Sigma) and goat-anti mouse Alexa-488 secondary antibody. Similarly, for endogenous AKAP150 we used polyclonal antibody against AKAP150 (Upstate) and goat-anti rabbit Alexa-488. For visualization of other PSD-95 and AKAP150 constructs anti-GFP polyclonal antibody (Abcam) and goat-anti rabbit Alexa 488-conjugated secondary antibody were used. Synapses were simultaneously labeled using either monoclonal antibody against Bassoon (Stressgen) or rabbit anti-Bassoon polyclonal antibody (Synaptic Systems) depending on the species of the other primary antibody used. Depending on the species of the primary antibody against Bassoon, either goat anti mouse or goat anti rabbit Alexa 568 was used to visualize Bassoon puncta. All primary antibodies were applied overnight at 4°C and all secondary antibodies were incubated for 1 hr at 37°C.

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