Supplementary Methods (see also 1)

Cell culture, GluR2, NR1 and synapse staining

Hippocampal neurons from 18 day old rat embryos were cultured until 9-11 DIV on glass coverslips following the Banker technique as previously described2 (density of ~5000 neurons/cm²). First, images of neurites were taken by differential interference contrast (DIC) to ascertain specific synaptic and receptor localization. Then, to label synapses, neurons were incubated for 1-2 min at 20°C with 1 nM Mitotracker (Deep Red-Fluorescent Mitotracker, Molecular Probes, Leiden, The Netherlands), a dye that does not require cell depolarization as compared to the commonly used FM1-43 dye. Cy3 was coupled to the antibodies at a mean ratio of 1:1 using the Cy3 mono-reactive dye pack (Amersham Biosciences, UK). We used the mouse monoclonal anti-GluR2 (BD Pharmingen, California, USA) and affinity-purified rabbit polyclonal anti-NR1 pan antibodies1,2,3 that are both directed against extracellular epitopes of the GluR2 and NMDAR-1 subunits respectively. Then, neurons were incubated for 10 min at 37°C with anti-GluR2-Cy3 or anti-NR1-Cy3. After a few rinses, coverslips were mounted in a custom chamber with culture medium at 37°C. For the high KCl conditions, neurons were incubated with 40 mM KCl for 2 min at 20°C after synaptic and antibody labelling. For the TTX conditions, neurons were incubated with 1 µM TTX for either 10 min or 48 h at 37°C before synaptic and antibody labeling. For these recordings, the recording medium contained 1 µM TTX. For the long-lasting 48 h treatment, TTX was added at 8 DIV and neurons were tested at 10 DIV. All data (recording session) were taken within 20 min following primary antibody incubation to minimize endocytosis. For the TPA treatment, neurons were treated after synaptic and antibody labelings with either 100 nM TPA or with the inactive analogue, 100 nM...
4-α-PHR (isophorbol) for 5 min at 20°C. The recording medium contained the same
dose of TPA or 4-α-PHR used for the initial treatment. Calculation of the median
diffusion was carried out on at least 2-4 different cultures, which correspond to
approximately 25-50 different dendritic areas. Because antibody cross-linking may
lower the diffusion of NMDARs, we compared the NMDAR diffusion distribution using
different concentrations of anti-NR1 antibodies (data not shown). All diffusion
distributions were similar suggesting that antibody cross-linking did not interfere with
receptor diffusions.

Microscopy and single-molecule detection

A custom wide field single-molecule fluorescence inverted microscope equipped with
a 100x oil-immersion objective (NA = 1.4) was used (IX70 Olympus, Bordeaux,
France). The samples were illuminated for 30 msec at a wavelength of λ = 532 nm by
a frequency doubled YAG laser (Coherent INC., Les Ulis, France) at a rate of 15 Hz.
Use of a defocusing lens permitted illumination of a surface of 20 x 20 µm² with a
mean illuminating intensity of 3 kW/cm². An appropriate filter combination (DCLP550,
HQ600/75, Chroma Technology, Brattleboro, USA) permitted the detection of
individual fluorophore by a CCD camera system (Micromax, Princeton Instruments,
Trenton, NY, USA). Using the same excitation path with another filter combination
(DCLP650, HQ675/50, Chroma Technology, Brattleboro, USA), Red Deep
Mitotracker (Molecular probes, Leiden, The Netherlands) was excited with the λ =
633 nm line of a He-Ne laser (JDS Uniphase, Manteca, CA, USA) at an illuminating
intensity of 7 ± 1 kW/cm². We imaged and resolved discrete fluorescence spots¹³⁻⁵.
 Fluorescence spots exhibit one-step photobleaching and not gradual decay as for
ensemble photobleaching. The width of these spots corresponds to the point-spread function of the microscope and the signal originating from 350 ± 150 counts per 30 ms. Thus, only those fluorescence spots that have all the hallmarks of individual fluorescent molecules bound to GluR2 or NR1-containing receptors were retained for analysis. Only trajectories containing at least 4 points were retained. We calculated the instantaneous diffusion coefficient, \( D \), for each trajectory, from linear fits of the first 4 points (first 3 points for trajectory of 4 frames) of the mean-square-displacement versus time function using \( \text{MSD}(t) = \langle r^2(t) \rangle = 4Dt \). The two-dimensional trajectories of single molecules in the plane of focus were constructed by correlation analysis between consecutive images using a Vogel algorithm.

**Determination of Endocytosis rate and impact on mobility measurements**

Endocytosis rate was measured by immunohistochemistry. Briefly, live neurons were first incubated for 10 min with either the anti-GluR2 (BD Pharmingen) or anti-NR1 antibody at 37°C, washed, and then either fixed immediately with a 4% (w/v) paraformaldehyde solution (time zero) or incubated for 20 min at 37°C before fixation. To quantify surface versus endocytosed receptors, fixed neurons were first incubated for 45-60 min with 10 µg/ml with the secondary antibody, i.e. Alexa568 anti-mouse Ig or 10 µg/ml Alexa568 anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands) to saturate cell surface bound primary anti-GluR2 and anti-NR1 antibodies respectively. Neurons were then permeabilized with 0.3% (v/v) Triton (X100) and incubated for 30 min with 5 µg/ml of the appropriate secondary antibodies as above to reveal endocytosed GluR2 and NR1 subunits respectively. Images were quantified using Metamorph Software (Universal Imaging, Downingtown, PA).
Control experiments\textsuperscript{1} (data not shown) established that this procedure adequately specifically stains respectively surface and internalized receptors.

We found that 36 ± 10% of AMPARs ($n = 6$ neurons) and 17 ± 4% of NMDARs ($n = 6$ neurons) were endocytosed. This finding shows that the endocytosis rates of AMPAR and NMDAR are significantly different ($P < 0.05$, $t$-test). The extent of endocytosis is in agreement with other, earlier reports\textsuperscript{1,8,9}.

Mobility recordings were performed regularly from 0 to 20 min while the endocytosed pool was estimated at the end time, i.e. 20 min. Thus on average 18% of our recorded AMPARs (8.5% NMDARs) are endocytosed receptors. Unfortunately, we have no experimental way to determine for a given labeled receptor on live cells whether it is on the cell surface or internalized. However, we previously showed that endocytosed AMPARs pertain to the immobile pool of receptors\textsuperscript{1}. Since immobile AMPARs represent themselves 47% of the total receptors, the uncertainty induced by endocytosis is, in our control experimental conditions, in the order of 8%. This uncertainty is of 5% for NMDARs.

TPA treatment has been shown to increase GluR2-containing AMPAR endocytosis rate as well as to induce a redistribution of GluR2-containing AMPARs to synaptic sites\textsuperscript{10}. In another set of experiments, we thus investigated the effect of TPA treatment on AMPAR endocytosis. In agreement with previous report\textsuperscript{10}, TPA treatment (100 nM) significantly increased the amount of endocytosed AMPARs after 20 min incubation (control: 21 ± 8%, $n = 6$ neurons; TPA: 55 ± 11%, $n = 6$ neurons, $P < 0.05$, $t$-test). Thus, our measure of the AMPAR lateral mobility in this condition is likely biased by an increased proportion of internalized receptor, as we cannot distinguish between surface and internalised receptors. As above, the amount of bias...
is approximately half these values. Thus, about 10.5% and 27.5% of recorded receptors are likely endocytosed in control and TPA mobility measurements, respectively. We expect a 17% increase in the percentage of immobile receptors due to endocytosed receptors. We found in mobility experiments that TPA increased the percentage of immobile receptors by 25% and decreased the mean diffusion coefficient of mobile receptors by 43% (from 0.39 in control conditions to 0.22 µm²/s in the presence of TPA, \(n = 98\) and 61, respectively). Thus TPA both increases AMPAR endocytosis and decreases surface mobility.

In another condition, KCl treatment (40 mM, 2 min), that increased the AMPAR lateral diffusion, we found no significant change in the endocytosis rate (control: 21 ± 8%, \(n = 6\) neurons; KCl: 22 ± 5%, \(n = 6\) neurons, \(P > 0.05\), t-test).

**Supplementary Method References**