Supplementary Materials for paper:

Intracellular Ca^{2+} and not the extracellular matrix determine surface dynamics of AMPA-type glutamate receptors on aspiny neurons

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Content:

Materials and Methods

Primary neuronal cultures and transfection

Dissociated hippocampal cultures were prepared from E18 Sprague-Dawley rats or GAD65-GFP mice as described previously (Kaech and Banker 2006). Neurons were plated at a density of 300 x 10^3cells/ml on poly-L-lysine (Sigma) pre-coated cover slips. Cultures were maintained in serum free neurobasal medium supplemented with B27 and 4mM GlutaMax at 37°C in a humidified atmosphere (95% air and 5% CO_2). Astroglial subcultures were prepared from cortex of P0 to P2 rats and cultivated to confluent monolayer for one week before plating of neurons. Glia proliferation was stopped by AraC added at 4 day in vitro (DIV). All chemicals used for neuronal cultures were obtained from Invitrogen, unless indicated otherwise. All experimental procedures were approved by the local Committee for Ethics and Animal Research (Landesverwaltungsamt Halle, Germany). Neurons were transfected using Effectene kit at 5-7DIV with Homer 1c::GFP or Homer 1c::DsRed for electrophysiology and single particle tracking (SPT) or co-transfected with Homer 1c::DsRed and pHGFP::GluA1 for FRAP experiments (Quiagen) as previously described (Frischknecht, Heine et al. 2009).

Immunostaining, receptor cross-link, hyaluronidase treatment

Protocol for immunostainings is described previously (Heine, Groc et al. 2008). Following primary antibodies (AB) were used: polyclonal rabbit against extracellular N-terminal of GluA1, monoclonal mouse against N-terminal of GluA2 (both 0.5mg/ml, 1:500, Agro-bio, Bordeaux, France), polyclonal mouse GAD65 (1mg/ml, 1:1000, Abcam, Cambridge, UK), polyclonal rabbit Homer 1c (0.5mg/ml, 1:500, Synaptic Systems, Göttingen, Germany). Secondary goat anti mouse and donkey anti rabbit AB (cy3 conjugated, 1.5mg/ml, 1:1000) were purchased from Dianova (Hamburg, Germany). For cross-link (X-link) of GluA1 containing AMPARs, neurons were incubated with anti GluA1 (1:100) for 10 min followed by 10 min incubation with the secondary AB (1:500). After 3 times wash neurons were used to examine synaptic currents. For live staining of the ECM cultures were incubated overnight with fluorescein labeled Wisteria floribunda agglutinin (WFA, 2mg/µl, 1:100, Vector Lab, Burlingame, USA), which selectively stains chondroitinsulfate chains of proteoglycans aggrecan and brevican. Stainings were examined with upright microscope Zeiss with Apochromat x63/1.4 oil objective. Synaptic localization of GluA1 and GluA2 were identified by colocalization with Homer 1c. Images were acquired using MetaMorph software (Visitron Systems) and processed in ImageJ and custom made software OpenVeiw.

For enzymatic degradation of the ECM cultures (DIV21-29) were incubated with hyaluronidase (HYase, Sigma) either overnight (200U/5ml, 12 hours) or acutely (500U/5ml, 20 min) at 37°C prior to observations. Numbers of cultures used in each experiment are mentioned in text.

Fluorescent Recovery after Photobleaching (FRAP)

The set-up and methods to analyze fluorescence recovery were described previously (Frischknecht, et al. 2009). Hippocampal neurons were transfected with GluR1 or GluA2::phluorin and DsRed::Homer1c at DIV5 and processed at DIV18-21. FRAP experiments were performed on GluR1 or GluA2::phluorin at synaptic contacts identified by DsRed::Homer1c and at extrasynaptic sites. Briefly, fluorescence was excited using a DG4-fluorescent lamp (Sutter, USA) controlled by MetaMorph software (Universal Imaging, USA). To photobleach locally multiple AMPAR populations in an average distance of about 20 µm a point scanning device (UGA 40) in combination with a 488 nm laser diode (Rapp Opto Electronic, Hamburg, Germany) was used applying brief pulses of 50-100 ms duration and maximal power. Recovery from photobleach was monitored by consecutive acquisition at 10 Hz acquisition rate up to 5 min. Recovery curves were corrected for continuous photobleach and background noise as described elsewhere (Axelrod, Koppel et al. 1976). Bleached area was 2µm^2.

Single particle tracking of AMPA receptors and analysis

Quantum dots (QD) 655 Goat F(ab')2 anti-Rabbit IgG Conjugate (H+L) highly cross adsorbed were bought from Quantum Dot corporation (Life Technology, USA). QDs (1µl, 0.1 µM) were diluted in 9 µl PBS then blocked by adding 1 µl casein 15 min before use. Neurons were incubated in culture medium with either primary rabbit anti-GluR1 antibody (1:100) for 5 min followed by 2-3 min incubation with QD solution (final dilution 0.1 nM) at 37°C or with primary anti-GluA2 antibody (1:100) conjugated with cy5. In some experiments neurons were exposed to HYase (500U/5ml) or BAPTA-AM (50 µM; Invitrogen) for 20 min at 37°C prior to incubation with primary antibody. After 3 times wash in extracellular solution (see electrophysiology) enriched with 0.5% BSA (Sigma) cells were imaged at 37°C in a closed chamber mounted onto an inverted microscope Olympus IX71 equipped with oil objectives x60/1.35 NA or x100/1.3 NA. QDs and Homer1c::GFP labeling were detected by using a Xenon lamp (Polychrom 3000, TILL-Photonics, Germany, excitation filter 545-580 or 472/30 and HC 556/20 for QD and emission filters 512/630-25 and HC655/15 respectively). Signals were recorded with a back-illuminated thinned CCD camera Luca Andor (Andor Technology, Belfast, UK). GluA1-receptors labeled with QD and GluA2-cy5 subunits were imaged on dendritic regions selected by transfection with Homer1c at 30 Hz sampling rate with 500 consecutive frames up to 25 min of total experimental time.

The tracking of single QDs was performed with Palm Tracer based on MetaMorth software (Visitron Systems). Single QDs were identified by their blinking fluorescent emission and sub trajectories of the same QDs were analyzed as individual trajectories. Mean squared displacement (MSD) were computed for all trajectories of at least 10 frames. Instantaneous diffusion coefficients (D_{inst}) were calculated by linear fit of the first 4 points of the MSD plots versus time interval. Particles with D_{inst} smaller than 0.001μm²/s were considered as immobile according to recordings of fixed QDs. The resolution precision was 50nm. GluR1- and GluR2-containing AMPARs displayed similar properties.

Electrophysiology

Whole-cell patch clamp recordings were performed on aspiny and spiny neurons after 17-26DIV under perfusion (1.5–2 ml/min) with extracellular solution, containing (in mM): 145NaCl, 5KCl, 2$CaCl_2$, 2$MgCl_2$, 10D-glucose, 10Hepes/NaOH, pH 7.4 using EPC10 patch-clamp amplifier (HEKA, Germany). Data were filtered at 3 kHz and sampled at 10 kHz using Patch Master 2.32 software (HEKA, Lambrecht, Germany). Patch pipettes from borosilicate glass (BM150F-10P BioMedical Instruments, Germany) had resistances of 3.5–6 MΩ, when filled with intracellular solution (in mM): 130 K-gluconate, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 0.1 EGTA, 2 Mg-ATP, 0.3 Na-GTP, pH 7.2-7.3. Action potentials (AP) were characterized in current clamp mode at room temperature; measurements were not corrected for a liquid junction potential. The resting membrane potential was measured directly after establishment of the whole-cell configuration, than hold at –60 mV.

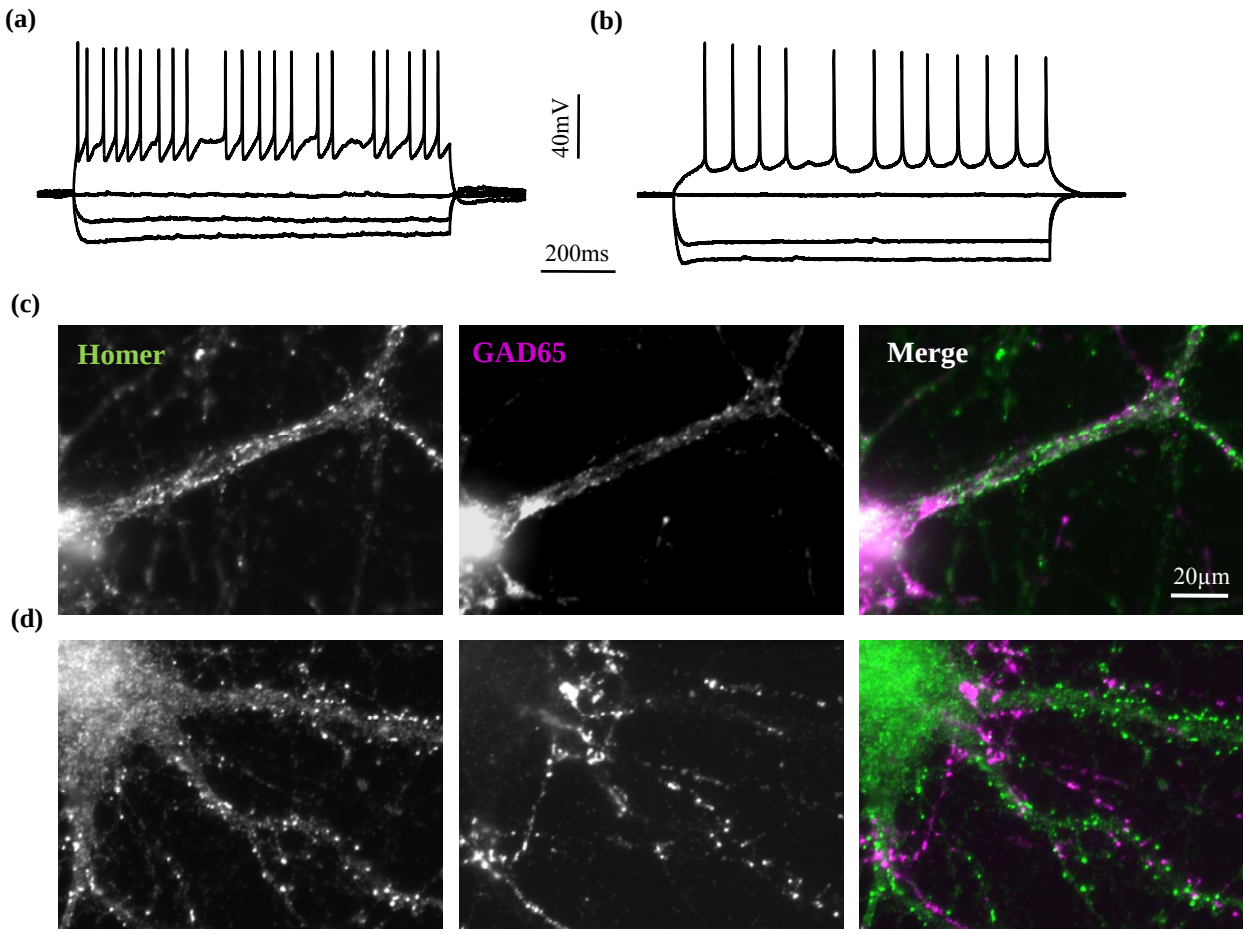
Evoked excitatory postsynaptic currents (eEPSC) and miniature postsynaptic currents (mEPSC) were recorded in voltage clamp mode from a holding potential of -60mV with an intracellular solution containing (in mM): 115 CH3CsSO3, 10 CsCl, 5 NaCl, 20 TEA chlorides, 10 HEPES, 0.6 EGTA. NMDA receptors antagonist D-2-amino-5-phosphonopentanoic acid (APV, 25 μM; Tocris, Bristol, UK), antagonist of GABA A receptors bicucullinemethiodide (BMI, 10 μM; Abcam, Cambrige, UK) and blocker of sodium channels tetrodotoxin (TTX, 1 μM; Tocris, Bristol, UK) were added to the bath in order to isolate AMPA currents and block spontaneous activity. AMPA eEPSC were evoked by local iontophoretic application of sodium glutamate (Glu) using an amplifier from NPI electronics (Tamm, Germany) and acquired at 30-33°C. Application pipettes had resistances of 50-90 MΩ when filled with Glu 150 mM, pH 7.4. A retaining current of 10-50 nA was applied to keep Glu inside the pipette. Glu was applied by current pulses of 150 - 300 nA, 1 ms with inter-stimulus intervals (ISI) of 20, 50, 100, 200, 500 and 1000 ms. Recovery from desensitization was reconstructed by plotting paired pulse ratios (PPR) against ISI. PPR were calculated as amplitude ratio of the second eEPSC to the first eEPSC averaged from 10 responses. Rectification index (RI) was calculated using formula: $(I_{+40}-I_0/I_0-I_{-60})x6/4$, where $I_{+40,}$ I_0 and I_{-60} are amplitudes of eEPSC measured at holding potentials of +40mV, 0mV and -60mV respectively. In some experiments philanthotoxin 433 (PhTx, 10 μM, Sigma) blocker of CP-AMPARs or kynurenic acid (Kyn, 500 μM, Sigma)

competitive blocker of AMPAR were applied to the bath. Only patches with the series resistances <15 MΩ were analyzed. Analysis of action potential properties, mEPSCs and eEPSCs were carried out using Mini Analysis Program (v6.0.3, Synaptosoft, USA) and Fitmaster software (HEKAv2x32, Germany) as described previously (Klueva, Meis et al. 2008). In control conditions 200 mEPSCs were analyzed 1 min after establishment of whole cell configuration.

Statistics

Data expressed as mean±SEM or as median and inter quartile range (IQR, 25%/75%). Statistical significances were tested using Graph Pad Prism (GraphPad Software v5.0, USA). Statistical tests are indicated within the figure descriptions. Indications of significant differences correspond to p-values: $p< 0.05$ (*), $p < 0.005$ (**) and $p < 0.001$ (***).

Figure S1.



Characterization of aspiny and spiny neurons in 3 weeks old hippocampal cultures.

(a, b) Voltage traces recorded in an aspiny (a) and a spiny (b) neurons during hyperpolarizing and depolarizing current steps (-250, -150, 0, +100 pA from bottom to top). Note higher firing frequency of action potentials in aspiny neuron. Resting membrane potential, action potential amplitude, half width and thresholds were not different between groups, although input resistance were was significantly lower in aspiny neurons (suppl. Tab. S1). (c, d) Immunocytochemical staining for Homer 1c (green) and GAD65 (magenta) reveal non-overlapping pattern in hippocampal aspiny (c) and spiny (d) neurons. Note positive immunoreactivity for GAD65 in soma of aspiny neurons confirming that they are GABAergic interneurons. Scale bar for all pictures shown in (c).

Figure S2.

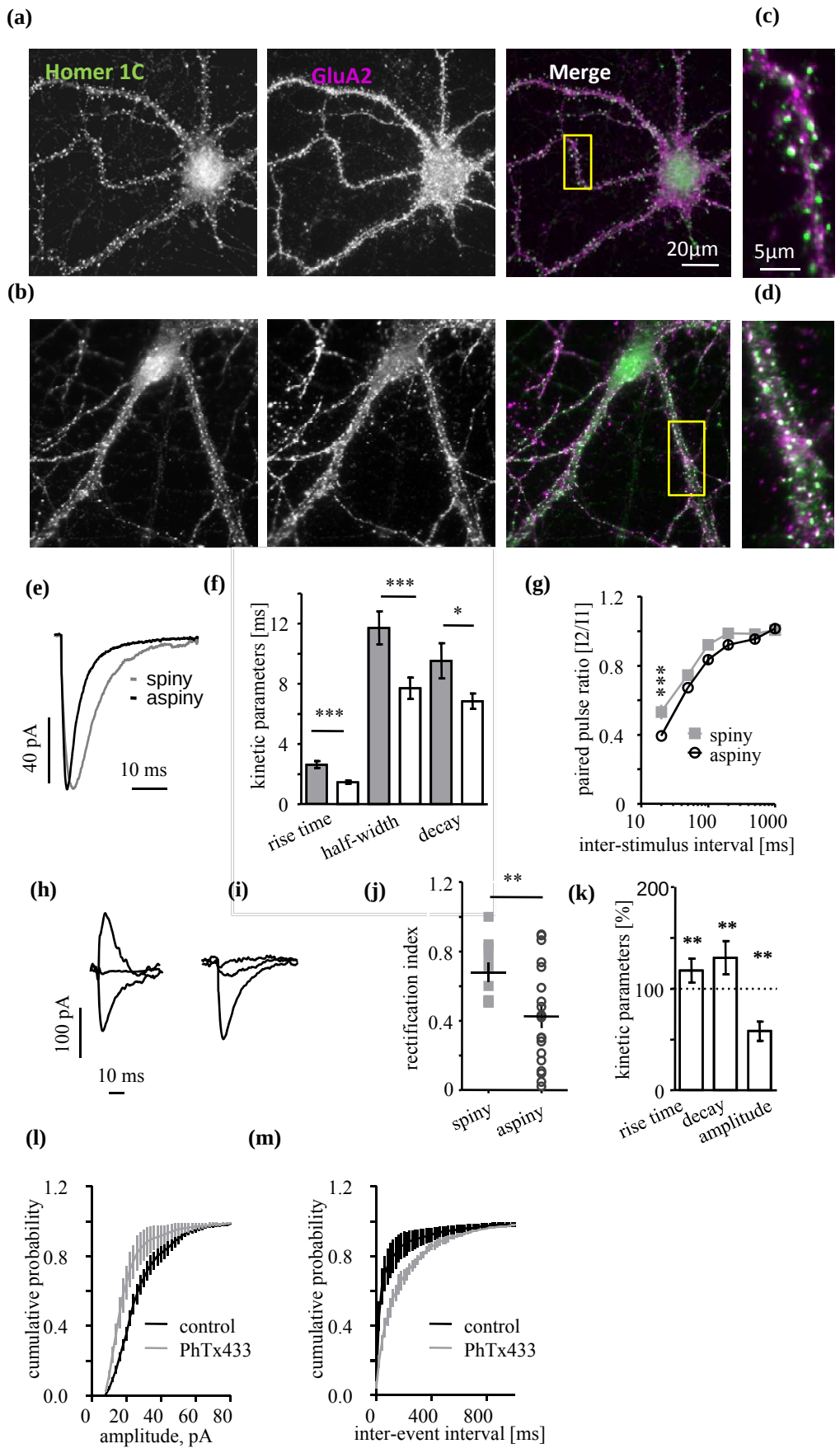

(a)
Homer 1C
GluA2
Merge
20µm
(c)
5µm
(b)
(d)
(e)
spiny
aspiny
40 pA
10 ms
(f)
kinetic parameters [ms]
rise time
half-width
decay
(g)
paired pulse ratio [I2/I1]
inter-stimulus interval [ms]
spiny
aspiny
(h)
100 pA
10 ms
(i)
(j)
rectification index
spiny
aspiny
(k)
kinetic parameters [%]
rise time
decay
amplitude
(l)
cumulative probability
control
PhTx433
amplitude, pA
(m)
cumulative probability
control
PhTx433
inter-event interval [ms]

Figure S2.

Characterization of subunit composition of synaptic AMPA receptors in spiny and aspiny neurons in hippocampal cultures (>DIV21).

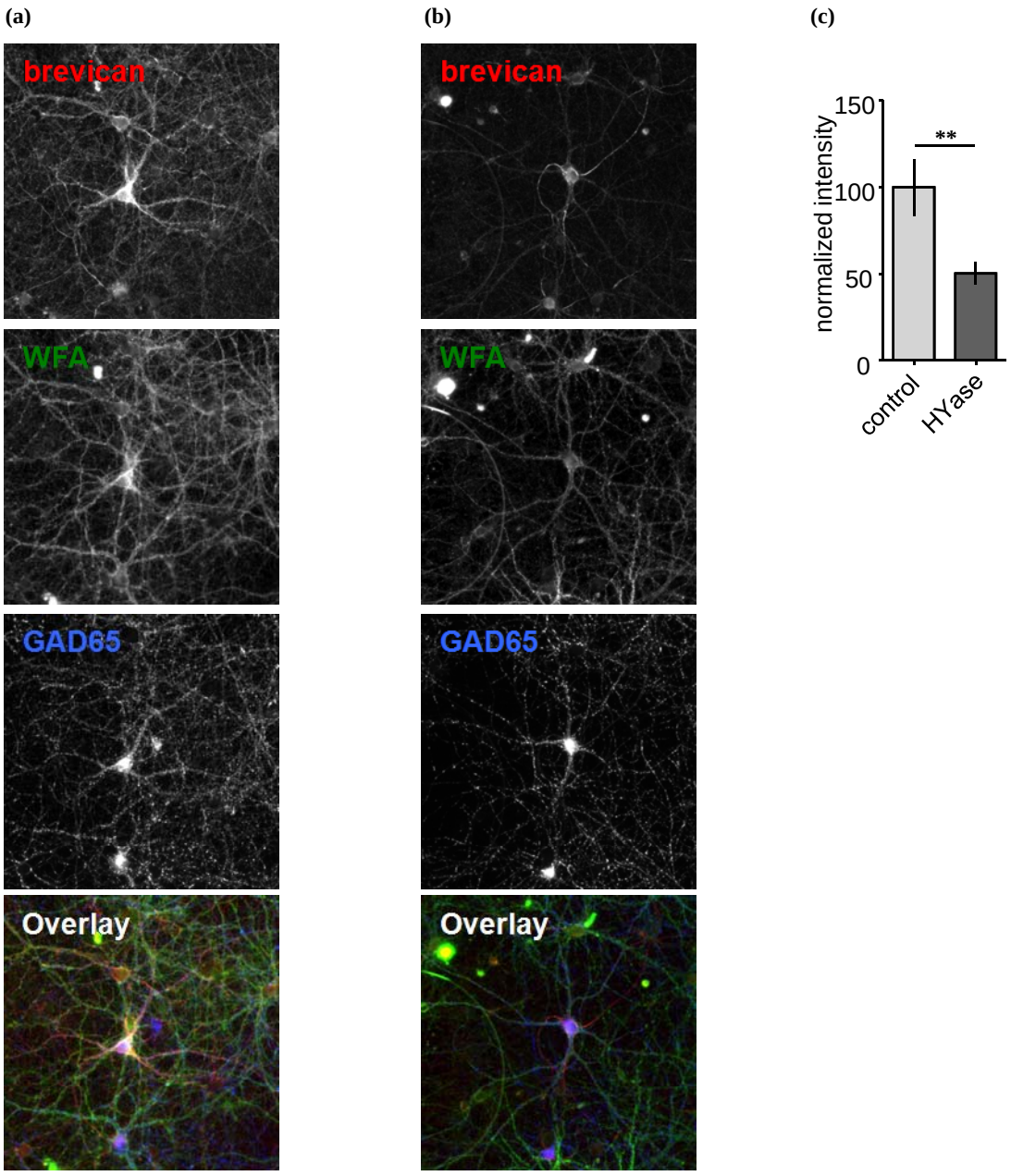
(a-d) Dual labeling of Homer 1c (green) and GluA2 (magenta) in a spiny (a) and an aspiny (b) neurons. Boxed region in (a) magnified in (c), boxed region in (b) magnified in (d). Scale bar for b shown in (a), scale bar for (d) shown in (c). (e) Superposition of eEPSCs elicited by iontophoretic application of Glu to spiny and aspiny neurons. (f) Mean±SEM of kinetic parameters of AMPA eEPSCs evoked by iontophoretic application of Glu to spiny and aspiny neurons ($p<0.05$ for decay, $p<0.005$ for rise time and half width, Mann-Whitney test. (g) Plot of recovery from desensitization of AMPARs on aspiny and spiny neurons as a function of inter-stimulus intervals (ISI). Recovery rates are different at ISI=20 ms, $p<0.01$, two-way ANNOVA with Bonferroni posttest. Error bars represent SEM. (h, i) Exemplified traces of whole cell current evoked by iontophoretic Glu application to spiny (h) and aspiny (i) neurons recorded at holding potentials of -60, 0 and +40 mV (from bottom to top). (j) Mean±SEM and individual values of rectification index (RI) in spiny and aspiny neurons; $p<0.01$, t-test. (k) Mean±SEM of rise time, half-width and amplitude of AMPA eEPSCs in aspiny neurons measured in the presence of PhTx 433 normalized to control values (dashed line at 100%), $p<0.01$, Wilcoxon sign rank test. (l, m) Cumulative distribution of amplitudes (l) and inter-event-intervals (m) of mEPSCs recorded in aspiny neurons in control conditions and 8 min after application of PhTx433 (n=5). PhTx433 decreased amplitude of mEPSCs ($p<0.05$) and increased the inter-event-intervals, $p<0.01$; Kolmogorov-Smirnov test.

Text to Figure S2.

To estimate the proportion of GluA1 and GluA2 subunits in AMPA receptors on spiny and aspiny neurons, cultures (DIV21-26) were stained using antibody against extracellular domains of GluA1 and GluA2 AMPAR subunits (Fig. 1 a-c, S2 a-d). Two-fold higher amounts of GluA1 were found at synapses of aspiny compared to spiny neurons (Fig. 1c, gray values aspiny 196.0±24.6, n=10; spiny 114.4±11.6, n=25; $p<0.01$), but there was no difference for GluA2 (Fig.1c, gray values aspiny: 150.7±14.2, n=15; spiny: 145.6±17.1, n=15; $p>0.05$).

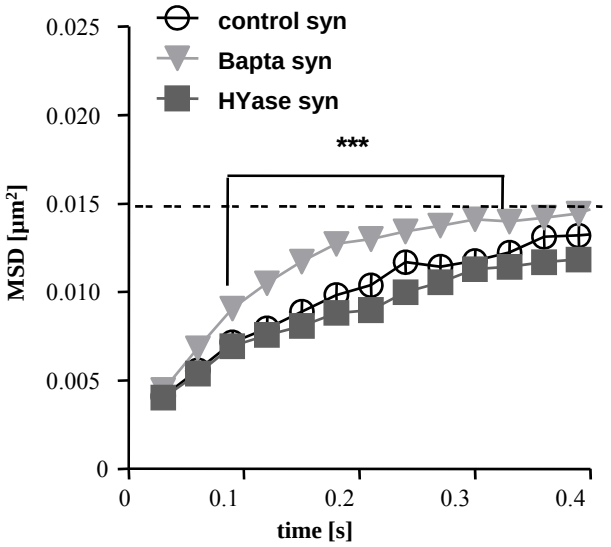
Using whole-cell patch clamp recordings we determined the proportion of GluA2 subunit containing AMPARs at glutamatergic synapses of aspiny and spiny neurons. AMPAR-mediated postsynaptic currents (eEPSC) were evoked by local fast iontophoretic application of glutamate to Homer1c::GFP-labeled synapses. AMPAR eEPSCs displayed faster kinetics in aspiny compared to spiny neurons (Fig. S2e, f, aspiny vs spiny for rise time: 1.46±0.10, n=26 vs 2.63±0.23, n=14, $p<0.001$; half width: 7.71±0.72 vs 11.72±1.10, $p<0.001$; decay time: 6.83±0.51 vs 9.53±1.16, $p<0.05$), whereas the amplitude of eEPSCs was not different between spiny and aspiny neurons, suggesting comparable density of AMPARs (aspiny vs spiny synapses -96.7±8.9 pA vs -92.1±15.63 pA, $p>0.05$). The rectification index (RI) was significantly smaller in aspiny than in spiny neurons (Fig. S2 h, i, aspiny vs spiny: 0.42±0.07 vs 0.68±0.06; $p<0.01$, Mann-Whitney test) reflecting larger proportion of either GluA2-lacking or unedited GluA2-containing AMPARs on aspiny synapses. Recovery from desensitization measured by changing the inter stimulus interval (ISI) was slower for aspiny than spiny glutamatergic synapses and was also evident in stronger depression observed at ISI <50 ms (Fig. S2 g, at ISI of 20ms aspiny vs spiny: 0.39±0.03, n=20 vs 0.53±0.04, n=13; $p<0.001$). In addition, we tested the sensitivity of synaptic AMPARs of aspiny neurons to philanthotoxin-433 (PhTx, 10 μM), which selectively blocks GluA2 lacking, calcium permeable AMPARs (CP-AMPARs) (Rozov, et al. 2012). Acute application of PhTx significantly reduced current amplitude to 58.1±9.4% of control value ($p<0.01$), increased rise time and prolonged the decay of evoked currents (Fig. S2 k, rise time: 117.7±11.9 % of control, $p<0.005$ and decay: 130.3±16.4%, $p<0.005$). Corresponding to the kinetic changes in the presence of PhTx433 we observed an increase of RI in aspiny neurons confirming the immunohistochemical data of a larger population of CP-AMPARs at aspiny synapses (Fig. S2l, control vs PhTx433: 0.63±0.08 vs 0.86±0.07, $p<0.01$). To assess the contribution of CP-AMPARs to basal synaptic transmission in aspiny neurons we recorded mEPSCs before and 8 min after exposure to PhTx433. PhTx433 reduced the amplitude and the frequency of mEPSCs to 75.6±7.5% (Fig. S2l, $p<0.05$) and 48.1±14.1 % of control values, respectively (Fig. S 2m, $p<0.01$) suggesting the presence of synapses exclusively expressing CP-AMPARs as indicated by the large scatter of the RI for aspiny glutamatergic synapses (Fig. S2 j). Kinetic parameters of mEPSCs were not changed after PhTx433 application (data not shown). These results demonstrate, that aspiny neurons in hippocampal cultures express to a higher extent GluA2 lacking AMPARs with faster kinetic and slower recovery from desensitization despite the similar abundance of GluA2 subunits in the neuronal membrane.

Figure S3.



Hyaluronidase (HYase) effectively digest extra cellular matrix (ECM) in hippocampal cultures.
(a) Anti-brevican and WFA fluorescein staining in primary hippocampal cultures at DIV21. Neurons were live-stained with WFA fluorescein (green) and after fixation colabeled with anti-brevican (red) and GAD65 (blue) to identify the main population of interneurons. Dense ECM wraps GAD65 positive neurons. (b) Similar staining as described for (a), 30 min after digestion of ECM by addition of 500 Units Hyaloronidase (HY) to the cultured medium. (c) Intensity of WFA staining in cultures after HY treatment normalized to control. Data are presented as mean ± SEM, 2 preparations, $p<0.01$, Wilcoxon singed rank test.

Figure S4.



Chelation of the intracellular Ca^{2+} selectively affects mobility but not confinement of GluA1 containing AMPAR in aspiny synapses measured in single particle tracking experiments.

Average mean square displacement (MSD) of synaptic GluA1 subunits of AMPARs under different conditions obtained from tracking of QD coupled to GluA1 antibody (BAPTA against control $p<0.005$ at time from 0.1 to 0.3 s, two-way ANNOVA). Dashed line shows the confinement area. Data are shown as mean ± SEM.

Table S1. Passive membrane properties and action potential characteristics in aspiny and spiny neurons.

	RMP (mV)	R_i (MΩ)	AP amplitude (mV)	AP half width (ms)	AP threshold (mV)	Max firing frequency (Hz)	Steady-state firing frequency (Hz)	AHP (mV)
aspiny N=8	-60.1±3.7	114.9±21.7	89.7±6.3	1.7±0.3	-43.9±3.6	103.2±6.3	44.4±7.7	-6.2±2.7
spiny N=11($^{\#}$5)	-57.9±1.4	193.1±18.9*	87.9±4.5	2.2±0.2	-49.5±2.4	78.7±6.9*	14.2±1.4**	-5.0±0.6$^{\#}$

RMP – resting membrane potential
R_i – input resistance
AP – action potential
AHP – afterhypopolarization

*-p<0.05, **-p<0.01, Mann-Whitney test

Table S2. Hyaluronidase has no effect on intrinsic properties and basal synaptic transmission in aspiny neurons.

	RMP (mV)	AP amplitude (mV)	AP half width (ms)	AP threshold (mV)	mEPSC ampl (pA)	mEPSC rise time (ms)	mEPSC decay (ms)	mEPSC frequency (Hz)
control N=7//8	-59.8±3.5	87.2± 6.4	3.2± 0.9	-40.9±4.3	-19.9±1.4	0.65±0.09	2.17±0.19	11.8±3.7
HYase N=8//8	-56.4±3.6	91.1±4.3	2.4±0.7	-40.4±4.2	-17.0±2.9	0.72±0.13	2.08±0.30	6.6±2.4

HYase - hyaluronidase
RMP - resting membrane potential
AP – action potential
mEPSC – miniature excitatory post synaptic current

References

Ashby, M. C., K. Ibaraki and J. M. Henley (2004). "It's green outside: tracking cell surface proteins with pH-sensitive GFP." Trends Neurosci **27**(5): 257-261.

Axelrod, D., D. E. Koppel, J. Schlessinger, E. Elson and W. W. Webb (1976). "Mobility measurement by analysis of fluorescence photobleaching recovery kinetics." Biophys J **16**(9): 1055-1069.

Frischknecht, R., M. Heine, D. Perrais, C. I. Seidenbecher, D. Choquet and E. D. Gundelfinger (2009). "Brain extracellular matrix affects AMPA receptor lateral mobility and short-term synaptic plasticity." Nat Neurosci **12**(7): 897-904.

Heine, M., L. Groc, R. Frischknecht, J. C. Beique, B. Lounis, G. Rumbaugh, R. L. Huganir, L. Cognet and D. Choquet (2008). "Surface mobility of postsynaptic AMPARs tunes synaptic transmission." Science **320**(5873): 201-205.

Kaech, S. and G. Banker (2006). "Culturing hippocampal neurons." Nat Protoc **1**(5): 2406-2415.

Klueva, J., S. Meis, A. D. de Lima, T. Voigt and T. Munsch (2008). "Developmental downregulation of GABAergic drive parallels formation of functional synapses in cultured mouse neocortical networks." Dev Neurobiol **68**(7): 934-949.

Rozov, A., R. Sprengel and P. H. Seeburg (2012). "GluA2-lacking AMPA receptors in hippocampal CA1 cell synapses: evidence from gene-targeted mice." Front Mol Neurosci **5**: 22.