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

Julia Klueva\textsuperscript{1,2,3}, Eckart D. Gundelfinger\textsuperscript{2,4,5}, R. Renato Frischknecht\textsuperscript{2,†} and Martin Heine\textsuperscript{1,†}

\textsuperscript{1}Research group, Molecular Physiology, \textsuperscript{2}Department of Neurochemistry/Molecular Biology, and \textsuperscript{3}Research group, Presynaptic Plasticity, Leibniz Institute for Neurobiology, Brennekestrasse 6, 39118 Magdeburg, Germany
\textsuperscript{4}Center for Behavioral Brain Science (CBBS), Magdeburg, Germany
\textsuperscript{5}Molecular Neurobiology, Medical Faculty, Otto von Guericke University, Magdeburg, Germany

The perisynaptic extracellular matrix (ECM) contributes to the control of the lateral mobility of AMPA-type glutamate receptors (AMPARs) at spine synapses of principal hippocampal neurons. Here, we have studied the effect of the ECM on the lateral mobility of AMPARs at shaft synapses of aspiny interneurons. Single particle tracking experiments revealed that the removal of the hyaluronan-based ECM with hyaluronidase does not affect lateral receptor mobility on the timescale of seconds. Similarly, cross-linking with specific antibodies against the extracellular domain of the GluA1 receptor subunit, which affects lateral receptor mobility on spiny neurons, does not influence receptor mobility on aspiny neurons. AMPARs on aspiny interneurons are characterized by strong inward rectification indicating a significant fraction of Ca\textsuperscript{2+}-permeable receptors. Therefore, we tested whether Ca\textsuperscript{2+} controls AMPAR mobility in these neurons. Application of the membrane-permeable Ca\textsuperscript{2+} chelator BAPTA-AM significantly increased the lateral mobility of GluA1-containing synaptic and extrasynaptic receptors. These data indicate that the perisynaptic ECM affects the lateral mobility differently on spiny and aspiny neurons. Although ECM structures on interneurons appear much more prominent, their influence on AMPAR mobility seems to be negligible at short timescales.

1. Introduction

AMPA receptors (AMPARs) are the principal excitatory neurotransmitter receptors in the brain. They are highly mobile within the neuronal membrane owing to lateral diffusion [1,2]. Diffusion and trapping by the postsynaptic scaffold are key factors controlling AMPAR numbers at the synapse, and thereby regulating synaptic strength [2,3]. Immobilization of diffusive receptors at synapses occurs mainly by direct or indirect interaction with intra- and/or extracellular molecules. Intracellular factors include PDZ domain-containing scaffold proteins such as PSD95 and SAP97, which bind AMPARs directly or via auxiliary subunits, e.g. TARPs [4,3]. PDZ binding is reversible and activity-dependent; high synaptic activity, which leads to elevated intracellular Ca\textsuperscript{2+} and subsequent CaMKII-dependent phosphorylation of stargazin, increases the affinity of AMPARs for PDZ domains and enhances their synaptic accumulation [6,7]. Furthermore, the spine itself may act as a diffusion barrier, because lateral diffusion is restricted at the spine neck, and AMPARs exchanged faster between synaptic and extrasynaptic compartments on aspiny neurons, as measured by fluorescence recovery after photobleaching (FRAP) [8].

As extracellular factors affecting mobility and clustering of AMPARs, components of the extracellular matrix (ECM) are discussed. The brain’s ECM is a meshwork of proteins of neuronal and glial origin [9]. Main components are...
chondroitin sulfate proteoglycans, including brevican and agrican that are coordinated by the glycosamine–glycan hyaluronan [10,11]. This ECM is found around most neurons and their synapses in the brain. While it has a loose appearance on excitatory forebrain neurons, it appears as dense, net-like structures around parvalbumin-positive interneurons, where the ECM forms so-called perineuronal nets [11,12]. Owing to its net-like appearance, the ECM has been postulated to define compartments on the neuronal surface that isolate synaptic contacts and control the lateral diffusion of AMPARs. Indeed, experimental removal of the ECM with the glycosidase hyaluronidase increased lateral diffusion and exchange of synaptic versus extrasynaptic AMPARs on principal neurons [13]. Physiologically, ECM removal was associated with decreased paired-pulse depression very likely owing to rapid exchange of desensitized synaptic for naive extrasynaptic AMPARs [13,14]. Thus, on spiny neurons, synaptic availability of AMPARs is defined by interplay between membrane-associated cytoplasmic scaffolds, i.e. the PSD, spine morphology and ECM-based surface compartments. Here, we wondered whether on aspiny interneurons the lack of spines as diffusion barriers might be functionally compensated by ECM structures. To test this, we analysed the influence of the ECM on lateral mobility and short-term plasticity of aspiny neurons.

2. Material and methods
A detailed description of chemicals and antibodies used in this study is provided in the electronic supplementary material.

(a) Neuronal cultures, fluorescence recovery after photobleaching experiments
Preparations of primary cultures from embryonic rat hippocampi (E18), their transfection with Effectene and matrix digestion procedure are described in the electronic supplementary material. Protocol for immunostainings has been described previously [13,14]. Protocol for single particle tracking (SPT) of AMPARs and its analysis are described in the electronic supplementary material. Set-up and methods to analyse FRAP were described previously [13].

(b) Electrophysiology
Whole-cell patch-clamp recordings were performed and analysed as described in the electronic supplementary material.

(c) Statistics
Data are expressed as mean ± s.e.m. or as median and interquartile range (IQR, 25%/75%). Statistical analysis was performed with Graph Pad Prism (GraphPad Software v. 5.0, USA). Statistical tests are indicated within the figure descriptions. Significant differences correspond to p-values: *p < 0.05, **p < 0.005 and ***p < 0.001.

3. Results
(a) Lateral mobility of GluA1 and GluA2 at aspiny synapses is not restricted by the extracellular matrix
The identification of aspiny glutamatergic synapses in our experiments is based on the co-localization of the scaffold protein Homer1 with AMPAR in spiny as well as aspiny neurons (figure 1a–c). In spiny neurons, Homer1 puncta accumulated in spine heads along the entire dendritic tree (figure 1a). In aspiny neurons, Homer1 was distributed in puncta along smooth dendrites and the soma (figure 1b). Co-staining of the surface population of AMPARs by specific antibodies against extracellular epitopes confirmed a higher abundance of GluA1 subunits in aspiny Homer-positive synapses, but no difference in GluA2-containing AMPARs (figure 1c). The majority of aspiny neurons represent GAD65-positive interneurons (electronic supplementary material, figure S1 and table S1). Electrophysiological characterization of the postsynaptic receptor composition revealed that aspiny neurons localize a substantial fraction of Ca²⁺-permeable AMPARs in their synapses as indicated by the inward-rectifying current–voltage relationship, confirming previous characterizations of the AMPAR population on GABAergic neurons (electronic supplementary material, figure S2) [15–17].

The influence of the ECM on the distribution and surface mobility of AMPARs in interneurons was probed in cultures that had been maintained for more than 21 days in vitro. At this age, dense nets of ECM were detectable around all neurons but were particularly dense around aspiny neurons (electronic supplementary material, figure S3). The ECM was removed with hyaluronidase (HYase) reducing Wisteria floribunda agglutinin staining to 50.3 ± 6.5% of control (electronic supplementary material, figure S3c). The digestion of the ECM by HYase occurred within the first 30 min after enzyme application and was not altered after overnight digestion as demonstrated previously for the mobility of AMPARs on glutamatergic neurons [13]. SPT on endogenous surface populations of GluA1- and GluA2-containing AMPARs was employed to test whether the pronounced ECM on interneurons affected the local AMPAR mobility. Antibodies against the N-terminal domain of GluA1 were labelled with quantum dots (QDs), and antibodies against GluA2 were labelled with ATTO647. QDs or ATTO647-molecules co-localizing with overexpressed Homer1c::GFP spots were considered as synaptic. The distribution of the instantaneous diffusion coefficients ($D_{\text{inst}}$) was shifted to smaller values for GluA1 and GluA2 at aspiny synapses compared with dendrites (figure 1d for GluA1, syn versus all, median: 0.008 μm² s⁻¹ IQR 0.003/0.021, 94 trajectories, versus 0.019 μm² s⁻¹ IQR 0.005/0.067, 606 trajectories from eight cells, six cultures p < 0.005; figure 1c for GluA2, syn versus all: 0.014 μm² s⁻¹ IQR 0.005/0.04, 494 trajectories, versus 0.019 μm² s⁻¹ IQR 0.006/0.056, 6454 trajectories, 12 cells, six cultures p < 0.005). The fraction of immobile GluA1 and GluA2 subunits at aspiny synapses and extrasynaptic locations was different with a larger fraction of immobilized GluA2-containing receptors in both compartments (figure 1d,e; 38.5%, 55 trajectories for GluA1 and 44.8%, 395 trajectories for GluA2 at synapses and 37.2%, 346 trajectories for GluA1 and 42.2%, 4614 trajectories for GluA2 at dendrites).

In contrast to spiny neurons [13], acute ECM removal with HYase did not change the mobility of synaptic or extrasynaptic GluA1 on aspiny neurons (figure 1f, after HYase synaptic, median: 0.011 μm² s⁻¹ IQR 0.004/0.030, 477 trajectories; all: 0.015 μm² s⁻¹ IQR 0.005/0.055, 2692 trajectories, from five cultures). Similarly, endogenous GluA2-containing receptors at shaft synapses were not affected by HYase treatment, but decreased in their mobility outside synapses (figure 1g, after HYase synaptic, median: 0.013 μm² s⁻¹ IQR 0.003/0.053, 86 trajectories, three cultures; all: 0.011 μm² s⁻¹ IQR 0.004/0.025, 829 trajectories, two cultures). Higher mobility at extrasynaptic locations than at synapses was preserved after matrix
degradation for GluA1 \( (p < 0.005) \). These data suggest that ECM has either no strong impact on the local mobility of the endogenous population of GluA1- and GluA2-containing receptors or AMPARs on aspiny synapses have different properties.

(b) Short-term synaptic plasticity in aspiny interneurons

To examine AMPAR properties before and after ECM digestion, we probed kinetic parameters found to be affected by ECM digestion [13]. Postsynaptic AMPARs were probed before and after ECM removal by fast iontophoretic Glu application on Homer-positive aspiny synapses. HYase treatment overnight did not alter membrane properties and basic synaptic transmission. Membrane potential, action potential amplitude and width as well as kinetics and frequency of mEPSCs were unchanged after matrix removal (electronic supplementary material, figure S2). Acute incubation of cultures with HYase before patch-clamp experiments also had no effect on the membrane properties, ruling out homeostatic effects of long-term incubation (not shown). Further, we wondered whether long-term ECM...
removal influences the organization of aspiny glutamatergic synapses. However, no change in rectification index (RI) was observed when comparing synapses before and after ECM digestion (figure 2a, aspiny control versus HYase: 0.42 ± 0.07, n = 19 versus 0.37 ± 0.07, n = 15, p > 0.05). The high variability of the RI points to a quite heterogeneous...
population of AMPARs expressed in aspiny neurons under our culture conditions, which probably masks ECM-induced changes in the RI. In order to reduce the variability, we used as a control a mouse line expressing GFP under the GAD-65 promoter to identify interneurons. Here, the RI was less variable; however, ECM digestion also had no significant effect (control versus HYase: 0.17 ± 0.04, n = 10 versus 0.23 ± 0.08, n = 3, p > 0.05). Labelling the surface population of GluA1- and GluA2-subunits on aspiny neurons also did not reveal different AMPAR densities before and after digestion (GluA1: 118.1 ± 8.7% of control after HYase, n = 14, p = 0.19; GluA2: 115.4 ± 8.8% of control after HYase, n = 21, p = 0.49). Next, we tested the effect of ECM on the recovery of AMPARs from desensitization. Overnight digestion did not affect recovery from desensitization of AMPARs (figure 2b), indicating that, in contrast to spiny neurons [13], ECM does not influence recovery from desensitization of synaptic receptors or lateral exchange with naive extrasynaptic receptors on aspiny neurons. To exclude effects caused by steady-state desensitization of AMPARs and to focus on the population of activated AMPARs by glutamate iontophoresis, we applied the weak competitive antagonist kynurenic acid (Kyn). Here, only populations of AMPARs exposed to glutamate concentrations that are sufficient to replace Kyn are activated. In the presence of Kyn, recovery from desensitization was significantly slower for synaptic receptors compared with extrasynaptic AMPAR, confirming our SPT experiments that indicated lower mobility inside synapses than outside (figure 2d).

The existence of a mobile population inside and outside the synapse might still allow exchange of receptors between compartments. To prevent this, we immobilized surface AMPARs by cross-linking [14,18]. Cross-linking of GluA1-containing receptors with antibodies before (X-link) and after acute matrix digestion (HYase + X-link) did not alter paired-pulse ratio (PPR) or recovery from desensitization (figure 2b). None of the treatments affected amplitude or kinetics of the evoked excitatory postsynaptic current (eEPSC; figure 2b inset and 2c) confirming the absence of direct effects of HYase, X-link or the combination on kinetic properties of AMPARs. The variability of the RI in synapses of aspiny neurons may either mask the local dynamic fluctuation of the AMPAR population or simply reflect a rather rigid assembly and/or subunit composition of synaptic receptors in aspiny synapses. Our data indicate that synaptic AMPARs on aspiny neurons are highly confined and their mobility is not modulated by ECM despite the differences in ECM density in comparison with spiny neurons.

These observations argue against the hypothesis that the ECM acts as passive diffusion barrier on aspiny neurons. Nevertheless, we wondered whether an increase of the mobile population of AMPARs might uncover the ECM-mediated compartmentalization. To modulate the mobile AMPAR fraction and its local confinement, we overexpressed pHluorin-tagged GluA1 and GluA2 subunits, a manipulation known to induce an approximately twofold increase in the surface population of GluA1- or GluA2-containing AMPARs [14,19]. The properties of the pHluorin [20] allowed FRAP experiments to be performed to probe the mobility of surface-expressed GluA1- and GluA2-containing AMPARs.

Under these conditions, enzymatic removal of ECM with HYase significantly increased the recovery rate of GluA1:pHluorin and GluA2:pHluorin fluorescence in synaptic and extrasynaptic membrane compartments (figure 2c,f synaptic control versus HYase: GluA1: 48 ± 2%, n = 32 versus 58 ± 4%, n = 17, p = 0.021; GluA2: 46 ± 5%, n = 15 versus 60 ± 3%, p = 0.022 and dendritic control versus HYase: GluA1: 76 ± 3%, n = 27 and 86 ± 2%, n = 22, p = 0.014; GluA2: 66 ± 5%, n = 7 versus 78 ± 3%, p = 0.04, t-test). A similar increase in fluorescence recovery after matrix digestion was observed in spiny synapses [13] confirming the proposed impact of ECM composition on AMPAR surface dynamics. Some limitations of this approach have to be considered. First, the bleached area is determined by the diffraction limit of the microscope (usually ≥1 μm²) and hence larger than most postsynapses in cultured neuronal networks. Second, overexpression of fluorescence-tagged proteins induces higher surface dynamics of receptors [21]. Thus, we assume that modulation of the mobile fraction of AMPARs in aspiny neurons might be controlled by intracellular binding partners. In particular, the Ca²⁺ permeability of the AMPARs prevalent in aspiny neurons (figure 1c and the electronic supplementary material, figure S2) might cause a stronger confinement of the receptors and hence overrule the ECM-based membrane compartmentalization.

(c) Mobility of GluA1 on aspiny neurons is regulated by intracellular Ca²⁺

In spiny neurons, a transient increase of intracellular Ca²⁺ via uncaging or strong synaptic activation induces strong immobilization of AMPARs [1,7,14]. To test whether indeed intracellular Ca²⁺ fluctuations are responsible for the strong confinement of AMPARs on aspiny neurons, we either clamped the intracellular Ca²⁺ concentration by incubating cultures in BAPTA-AM or blocked the fraction of potentially calcium-permeable AMPARs by phytotoxin 433 (PhTx433) and monitored the mobility of the endogenous receptor populations on spiny and aspiny neurons using SPT. BAPTA increased the mobility of synaptic and extrasynaptic fraction of GluA1 on aspiny neurons (figure 2g, median of $D_{\text{mot}}$ for synaptic GluA1 control versus BAPTA-AM: 0.008 μm² s⁻¹ IQR 0.001/0.144, 94 trajectories versus 0.020 μm² s⁻¹ IQR 0.001/0.402, 111 trajectories after incubation with BAPTA-AM, p < 0.01, Mann–Whitney test and for all GluA1 trajectories: control versus BAPTA-AM: 0.019 μm² s⁻¹ IQR 0.001/0.605, 606 trajectories versus 0.038 μm² s⁻¹ IQR 0.001/0.630, 636 trajectories, p < 0.005) without affecting the confinement area (electronic supplementary material, figure S4). In spiny neurons, chelating GluA1 had no effect on the mobility of GluA1 subunits (figure 2h, p > 0.05). Blocking calcium-permeable AMPARs with PhTx433 also mobilized AMPARs inside synapses of aspiny and spiny neurons, but only altered the mobility of extrasynaptic AMPARs in spiny neurons (figure 2g,h, median of $D_{\text{mot}}$ for aspiny synaptic GluA1 + PhTx433: 0.019 μm² s⁻¹ IQR 0.005/0.069, 291 trajectories; median of $D_{\text{mot}}$ for spiny synaptic GluA1 + PhTx433: 0.017 μm² s⁻¹ IQR 0.007/0.061, 479 trajectories; for all aspiny GluA1 PhTx433: 0.020 μm² s⁻¹ IQR 0.006/0.062, 766 trajectories; for all spiny GluA1 PhTx433: 0.034 μm² s⁻¹ IQR 0.010/0.116, 1708 trajectories).

4. Discussion

Here, we report that aspiny glutamatergic synapses on interneurons in hippocampal cultures contain highly confined AMPARs, which are partially Ca²⁺-permeable. This Ca²⁺...
permeability might be responsible for the strong confinement within the synapse that is not influenced by interactions with the perisynaptic ECM and hence does not interfere with the AMPAR-mediated short-term plasticity in most of these synapses.

The majority of aspiny neurons in dissociated hippocampal cultures are interneurons [22, 23], expressing an AMPAR population (electronic supplementary material, figures S1 and S2) and auxiliary proteins different from those in spine-containing neurons [24, 25]. Depending on subunit composition and auxiliary proteins, AMPARs differ substantially in their properties, including rectification, desensitization and recovery from the desensitization [16, 24, 26]. Here, we confirm that receptors on aspiny neurons show on average faster postsynaptic AMPAR-mediated currents and a slower recovery from desensitization compared with spiny neurons (electronic supplementary material, figure S2), which is reflected in higher numbers of surface-expressed GluA1 subunits (figure 1). This suggests that a considerable fraction (approx. 50%) of AMPARs in aspiny glutamatergic synapses are Ca\textsuperscript{2+}-permeable as confirmed by the sensitivity to PhTx433 (electronic supplementary material, figure S2; [24, 27]). This Ca\textsuperscript{2+} permeability might exert the function of confining AMPARs to synapses and thus explain their limited surface dynamics which is insensitive to ECM removal (figure 1). Similar observations were reported for spiny neurons when GluA1 subunits were overexpressed and Ca\textsuperscript{2+}-permeable GluA1 homomers were introduced in the synapse [14]. This suggests that the ECM-based compartmentalization does not affect the local fraction of mobile receptors in small compartments such as the synaptic contact site defined by Homer staining of aspiny synapses. However, when GluA1 is overexpressed in aspiny neurons, the highly mobile population of AMPARs is increased [18, 21], and the ECM acts as a passive diffusion barrier as observed on spiny neurons [13]. Experimental immobilization by cross-linking of endogenous GluA1 receptors does not affect their kinetic properties, and confirms our interpretation that AMPARs in aspiny synapses are more confined than in spiny synapses. This is reminiscent of the behaviour of NMDA-type receptors in spiny synapses [28] and left us with the hypothesis that the local amount of Ca\textsuperscript{2+}-permeable AMPARs might determine the synaptic confinement. Control of intracellular Ca\textsuperscript{2+} by BAPTA-AM or block of the Ca\textsuperscript{2+}-permeable receptor fraction before tracking AMPARs supports this idea (figure 2g,i). In spiny neurons, we also observed a mobilization of GluA1-containing AMPARs after block of GluA2-lacking receptors (but not with BAPTA). As suggested by the variability of the RI (electronic supplementary material, figure S2), there is also a population of Ca\textsuperscript{2+}-permeable AMPARs expressed in spiny synapses, which might serve a confining function.

Interestingly, the RI and hence the population of Ca\textsuperscript{2+}-permeable AMPARs was highly variable among aspiny synapses, probably owing to the heterogeneity of interneurons, different innervating axons [17] or activity-driven changes in accessory subunit compositions [29–31]. A functional explanation for this heterogeneity could be the critical involvement of interneurons in tuning the input–output function of neuronal network activity. Contacts between principal neurons tune the threshold for plasticity, whereas changes in the excitability of interneurons change the gain of plastic changes [32]. How strongly such effects depend on the individual composition of postsynaptic receptor populations remains an open question. The scattering of RI was evident not only between different aspiny neurons, but was also observed between different synapses along an individual aspiny neuron. Whether this is caused by a single axon or different axons was not addressed. Fluctuations of the release probability of individual boutons from the same axon can occur [33] and this could lead to activity-driven shaping of AMPAR compositions as suggested in cerebellar neurons [29]. Accordingly, inputs from different presynaptic synapses might be integrated by the postsynaptic receptor composition and in turn tune the output function of this particular neuron.

Removal of the ECM can influence the receptor dynamics and local receptor density and exchange rate between synaptic and extrasynaptic receptors [13]. In aspiny synapses, another variable seems to be important, which could be the population size of Ca\textsuperscript{2+}-permeable AMPARs and hence their Ca\textsuperscript{2+}-dependent confinement. Binding to intracellular scaffolds and intracellular kinase activity depends on the intracellular fluctuation of free Ca\textsuperscript{2+} [1, 7, 14]. This strong confinement might fulfil two functions, first preserving the inhibitory tone (output function) within a neuronal network and second protecting the neuron from excessive Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+}-permeable AMPARs [34, 35]. Accordingly, the function of the ECM seems to be different on aspiny and on spiny neurons. Whereas in spiny neurons, AMPARs seem to be less confined by intracellular binding partners or auxiliary subunits the ECM can function as an obstacle, particularly for the extrasynaptic population, whereas the synaptic population remains unbiased by changes in ECM composition or density [13]. At aspiny synapses, the contributions of mobile AMPARs to modulate synaptic transmission seem to be much more strongly controlled by intracellular interactions and are less influenced by the ECM, at least on the timescale of seconds to minutes that was observed here, despite a much higher density of ECM-like structures around aspiny neurons.

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