Molecular determinants for the strictly compartmentalized expression of kainate receptors in CA3 pyramidal cells

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Distinct subtypes of ionotropic glutamate receptors can segregate to specific synaptic inputs in a given neuron. Using functional mapping by focal glutamate uncaging in CA3 pyramidal cells (PCs), we observe that kainate receptors (KARs) are strictly confined to the postsynaptic elements of mossy fibre (mf) synapses and excluded from other glutamatergic inputs and from extrasynaptic compartments. By molecular replacement in organotypic slices from GluK2 knockout mice, we show that the faithful rescue of KAR segregation at mf-CA3 synapses critically depends on the amount of GluK2a cDNA transfected and on a sequence in the GluK2a C-terminal domain responsible for interaction with N-cadherin. Targeted deletion of N-cadherin in CA3 PCs greatly reduces KAR content in thorny excrescences and KAR-EPSCs at mf-CA3 synapses. Hence, multiple mechanisms combine to confine KARs at mf-CA3 synapses, including a stringent control of the amount of GluK2 subunit in CA3 PCs and the recruitment/stabilization of KARs by N-cadherins.
There is an extreme anatomical and functional diversity of synapses connecting different populations of neurons. The segregation of different excitatory connections onto a single neuron is particularly striking in laminated structures such as the hippocampus. CA3 pyramidal cells (PCs) receive three types of glutamatergic inputs, which are precisely positioned along apical and basal dendrites, from the entorhinal cortex (perforant path), from recurrent CA3 collaterals (associational/commissural, A/C fibres) and from the dentate gyrus (DG) through mossy fibres (mf). Glutamatergic synapses from these different inputs greatly vary in their structural and functional properties. Mf inputs make synaptic contacts on proximal dendrites (PDs) of CA3 PCs (in the stratum lucidum) via ‘giant’ mf boutons with multiple glutamate release sites facing large postsynaptic structures called thorny exocrescences (TEs). In contrast A/C and perforant path synapses are of the single site/single spine type. Mf-CA3 synapses display a wide dynamic range of short-term plasticity and express NMDA (N-methyl-D-aspartate) receptor-independent presynaptic forms of long-term depression (LTD) and long-term potentiation (LTP)3.

The different families of ionotropic glutamate receptors (iGluRs), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors (AMPArs), NMDA receptors and kainate receptors (KARs) exist as multiple subtypes endowed with distinctive properties. The subcellular segregation of KARs in CA3 PCs provides a striking example of synaptic specification within a single neuronal population. KARs are tetrameric iGluRs composed of combinations of five subunits GluK1–GluK5. KARs regulate the activity of synaptic circuits at presynaptic and postsynaptic sites, through either ionotropic or metabotropic actions6–8. KARs are expressed and functional in principal neurons of CA1, DG and CA3, as well as in interneurons. However, postsynaptic KARs activated by synaptic release of glutamate has only been encountered in CA1 interneurons and in CA3 PCs. In CA3 PCs, stimulation of mf inputs but not of A/C inputs evokes excitatory post-synaptic currents (EPSCs) mediated by KARs (KAR-EPSCs), which contain the GluK2 subunit10,11. In addition, puff application of glutamate above the stratum lucidum, but not the stratum radiatum, activates KAR-mediated inward currents10. This segregation of KARs in the stratum lucidum is further supported by kainate-binding studies and by immuno-cytochemical labelling of GluK2 and GluK5, although these methods do not discriminate between pre- and postsynaptic KARs12,13. A precise evaluation of the subcellular localization of native KARs in CA3 PCs is made difficult by the lack of high-quality antibodies.

The molecular mechanisms underlying subcellular trafficking of KARs have been explored in heterologous systems and in dissociated neuronal cultures6,7, albeit in conditions that do not reproduce the strict subcellular segregation observed for native KARs in an in vivo situation. The subcellular trafficking and localization of neurotransmitter receptors in neurons are tightly regulated by association with interacting proteins. KAR subunits interact through their cytoplasmic carboxy-terminal domain (CTD) with a large array of proteins involved in the regulation of trafficking to the plasma membrane14–17. In addition, NETO1 and NETO2 (neutropin tolloid-like 1 and 2) act as auxiliary proteins assembling with KARs to control gating and pharmacology18–23. NETO1 contributes to postsynaptic KARs at mf-CA3 synapses20,22, but the effect of NETO proteins on KAR targeting is unclear19,24. NETO proteins do not appear to be responsible for the segregation of KARs to mf-CA3 synapses23.

N-cadherin, which participates in transsynaptic complexes to ensure the adhesion between synaptic membranes and organize the underlying multiprotein complex25, interacts with GluK2a through its CTD domain16. In transfected hereologous cell lines, activation of N-cadherin by ligand-covered latex beads recruits GluK2 to N-cadherin/β-catenin complexes15. Whether this interaction plays a role in the trafficking and stabilization of KARs at synaptic sites has not yet been explored.

Here we investigated the mechanisms underlying the confined subcellular expression of KARs in CA3 PCs. Both cell autonomous mechanisms and signals from afferent mfs through transsynaptic-synaptic signalling may be at play. We have used focal glutamate uncaging combined with electrophysiology to precisely characterize the functional expression of KARs and combined this functional approach with a semi-quantitative gene replacement strategy to identify molecular mechanisms for the subcellular segregation of KARs in CA3 PCs.
Figure 1 | Compartmentalized expression of KARs in CA3 PCs. (a) Schematic experimental setting. (b) Voltage-clamp recordings performed on CA3 PCs from acute and organotypic slices prepared from GluK2+/+ and GluK2−/− mice, in the presence of bicuculline (10 μM), D-AP5 (50 μM) and a low concentration of NBQX (150 nM), to limit polysynaptic activity. In all, average of 30 responses evoked at 3 Hz in the absence and in the presence of 25 μM LY303070, to isolate the mf-CA3 AMPAR-EPSCs and KAR-EPSCs, respectively. Insert, enlargements of isolated KAR-EPSCs. (c) Summary graphs for the relative amplitude of KAR-EPSCs versus AMPAR-EPSCs obtained in acute and organotypic slices prepared from GluK2+/+ and GluK2−/− mice. MF KAR/AMPA ratio, acute GluK2+/+: n = 6 cells; organotypic GluK2+/+: n = 8 cells; acute GluK2−/−: n = 5 cells; organotypic GluK2−/−: n = 12 cells; A/C KAR/AMPA ratio: acute GluK2+/+: n = 7 cells; organotypic GluK2+/+: n = 7 cells; 27 mice. (d) Confocal image of a CA3 PC transfected with GFP illustrating glutamate uncaging spots (MNI glutamate, 500 μM) and corresponding evoked KAR currents. Purple arrowheads represent time of uncaging (405 nm, 2 ms). Average of five responses evoked at 0.1 Hz at each uncaging site. (e,f) Sample traces illustrating currents evoked by glutamate uncaging (bicuculline 10 μM, D-AP5 50 μM and NBQX 150 μM) in GluK2+/+ organotypic slices in different compartments of CA3 PCs, soma (S), thorny excrescences (TE), proximal shaft (PS) and distal dendrites (DDs). Summary graph of the average of net amplitude of evoked currents. Uncaging sites: S, n = 3; TE, n = 7; PS, n = 3; DD, n = 10, from 4 cells; 2 mice. (g,h) Sample traces illustrating KAR currents evoked by glutamate uncaging in GluK2+/+ organotypic slices in different compartments of CA3 PCs. Graph summarizing the average net amplitude of evoked KAR currents. Uncaging sites: S, n = 7; TE, n = 10; PS, n = 5; DD, n = 10, from 10 cells; 6 mice. (i,j) Sample traces illustrating the lack of KAR currents evoked by glutamate uncaging in GluK2−/− organotypic slices in different compartments of CA3 PCs. Graph summarizing the average net amplitude of the evoked currents. Uncaging sites: S, n = 8; TE, n = 18; PS, n = 1; DD, n = 8, from 5 cells; 4 mice.

could only be evoked when the uncaging spot was localized above visually identified TEs (average amplitude 59.4 ± 13.2 pA, n = 10; Fig. 1g,h). No KAR current could be evoked when the laser spot was either positioned on the soma, on the shaft of PDs or on DDs. No KAR current was evoked in GluK2−/− organotypic slices (Fig. 1i,j) or in any compartment of CA1 PCs (Fig. 1d,e), further indicating that all other iGluRs were adequately blocked. In acute slices, functional mapping of KARs revealed a similar restricted pattern of expression (Supplementary Fig. 1f,g). These functional mapping results highlight a clear distinction between iGluR subtypes. AMPARs are readily expressed at synaptic and extrasynaptic sites, that is, on the soma and dendritic shafts. In contrast, KARs appear excluded from the soma and from the proximal and distal dendritic shafts, and are only activated when the laser is focused on TEs. With the current resolution of laser uncaging we cannot exclude the presence of KARs at perisynaptic sites within the TE, given the small spacing (300–400 nm) between the different postsynaptic densities (PSDs)28,29.

Semi-quantitative replacement of GluK2 in GluK2−/− mice. To gain insight into the mechanism of compartmentalization of KARs in CA3 PCs, we used a molecular substitution approach in combination with slice electrophysiology and glutamate uncaging (Fig. 2). In slices prepared from GluK2−/− mice, glutamate uncaging on TEs of CA3 PCs did not evoke any detectable
current, as expected. We then rescued functional KARs in GluK2−/− slices using single-cell electroporation of CA3-PCs. A main advantage of single-cell electroporation, with respect to viral infection, is the possibility to control the amount of cDNA transfected, in addition to being able to precisely target CA3 PCs. We transfected various concentrations (3, 10, 20, 40 and 80 ng μl⁻¹) of cDNA encoding GluK2a together with cDNA encoding soluble GFP as a reporter (Fig. 2 and Supplementary Fig. 2). Two to 3 days after electroporation of GluK2a cDNA at a concentration as low as 3 ng μl⁻¹, KAR currents evoked by focal glutamate uncaging onto TEs could be successfully recorded. The amplitude of KAR currents depended on the amount of cDNA transfected (mean amplitude: 80 ng μl⁻¹: 275.2 ± 64.4 pA; 40 ng μl⁻¹: 126.1 ± 41.3 pA; 20 ng μl⁻¹: 85.3 ± 22.1 pA; 10 ng μl⁻¹: 97.3 ± 9.5 pA; 3 ng μl⁻¹: 68.7 ± 12.3 pA; Fig. 2a–c). The average amplitude of native KAR currents in wild-type (WT) slices corresponded best to the lowest concentration of GluK2a cDNA (3 ng μl⁻¹). Importantly, with higher GluK2a cDNA concentrations, aberrant KAR currents were also evoked with focal glutamate uncaging onto the soma (Fig. 2b–d) and DDs (Supplementary Fig. 2), in stark contrast with the native situation (soma, mean amplitude: 80 ng μl⁻¹: 93.2 ± 29.7 pA; 40 ng μl⁻¹: 22.8 ± 4.1 pA; 20 ng μl⁻¹: 24.9 ± 9.6 pA; 10 ng μl⁻¹: 23.3 ± 4.2 pA).

In WT slices, KAR currents displayed slow decay kinetics (mean tau-weighted WT: 126 ± 17 ms), as a probable consequence of the heteromeric composition of native KARs and interaction with auxiliary subunits such as NETOs. In CA3 PCs of GluK2−/− mice transfected with GluK2a cDNA, the decay kinetics of KAR currents evoked by focal glutamate uncaging onto TEs was much faster than in native conditions for concentrations GluK2a cDNA of 10 ng μl⁻¹ and above (mean tau-weighted GluK2a 80 ng μl⁻¹: 18.1 ± 5.5 ms; 40 ng μl⁻¹: 46.6 ± 8.6 ms; GluK2a 20 ng μl⁻¹: 68.6 ± 9.4 ms; GluK2a 10 ng μl⁻¹: 71.9 ± 5.1 ms; Fig. 2e,f). The electroporation of 3 ng μl⁻¹ GluK2a cDNA restored decay kinetics to values not significantly different from WT (mean tau-weighted GluK2a 3 ng μl⁻¹: 103.4 ± 12.0 ms). In addition, KAR currents evoked in the soma for concentrations of GluK2a cDNA of 10 ng μl⁻¹ and above also displayed fast decay kinetics (mean tau-weighted GluK2a 80 ng μl⁻¹: 16.2 ± 2.1 ms). From a methodological standpoint, these semi-quantitative re-expression experiments clearly show that the amount of cDNA transfected and, consequently, of GluK2a protein is critical for a faithful rescue of KAR subcellular segregation similar to native conditions. The use of a low amount of GluK2a cDNA (3 ng μl⁻¹) restored the restricted functional expression of KARs in TEs, whereas transfection of a higher amount of cDNA led to the functional expression of KARs with ectopic localization and aberrant kinetics.

Recombinant homomeric GluK2 KARs display notably faster deactivation kinetics than heteromeric GluK2/GluK5 when activated by a short (1 ms) pulse of glutamate. Fast decay kinetics of ectopic KAR currents observed in conditions of overexpression of GluK2 may be due to the prevalence of homomeric GluK2. To test this hypothesis, we used UBP310 as an antagonist of recombinant heteromeric GluK2/GluK5 KARs and synaptic KARs, which spares homomeric GluK2 KARs. We observed that in WT slices, KAR currents evoked by glutamate uncaging on TEs was strongly inhibited by 3 μM UBP310 (mean...
% of inhibition: 86.5 ± 2.0%; Fig. 3) confirming that native KARs in CA3 PCs are likely to be heteromeric KARs. In CA3 PCs from GluK2−/− mice, following transfection of low concentrations of GluK2 cDNA (3 ng μl−1), KARs were also largely inhibited by UBP310 (mean % of inhibition: 81.6 ± 7.4%; Fig. 3). In contrast, following transfection of a higher amount of cDNA (80 ng μl−1), inhibition of KAR currents evoked by glutamate uncaging on TEs was largely attenuated (mean % of inhibition: 29.0 ± 12.3%) and this inhibition spared the fast decaying component of the response (Fig. 3). These results strongly suggest that following transfection of a high concentration of GluK2α cDNA, ectopic KARs with fast decay kinetics represent a pool of homomeric GluK2 KARs. These experiments also strongly support the notion that native KARs are heteromeric KARs. KAR currents can be successfully rescued in CA3-PCs albeit faithful replacement of that native KARs are heteromeric KARs. KAR currents can be reliably rescued at mf-CA3 synapses and were absent from other inputs, mimicking the distribution of native KARs. In addition, whereas the use of high cDNA concentration led to the expression of ectopic extrasynaptic KAR currents of large amplitude, the relative amplitude of KAR-EPSCs did not differ between the different concentrations of cDNA. Although the content of synaptic KARs is precisely controlled independently of the copy number of GluK2a cDNA expressed, the amount of extrasynaptic KARs depends on the concentration of cDNA electroporated. These observations imply that at synaptic contacts an active and specific molecular machinery precisely regulates the amount of KARs, possibly due to a limited number of slots to accommodate synaptic KARs at mf-CA3 synapses.

**Subcellular imaging of recombinant GluK2α in CA3-PCs.** Visualization of subcellular distribution of KARs neurons is limited by the lack of good antibodies. To circumvent this issue, we expressed GluK2α subunits bearing an amino-terminal SEP tag24 in CA3-PCs and labelled them with anti-GFP nanobodies conjugated to photostable organic dyes35. These small ligands (~3 nm) show rapid penetration into thick organotypic tissue in live conditions36, allowing robust staining of surface SEP–GluK2a. Specifically, we electroporated SEP–GluK2a at cDNA concentrations of 3 or 80 ng μl−1 in CA3 PCs of GluK2−/− slices, thus replacing endogenous KARs by SEP–GluK2a. KAR currents evoked by focal glutamate uncaging after expression of SEP–GluK2a (3 and 80 ng μl−1) were of similar amplitudes as that observed when GluK2α-WT was expressed (Supplementary Fig. 3). Moreover, mf-CA3 synaptic KARs were faithfully rescued after electroporation of SEP–GluK2a (3 ng μl−1 cDNA; Supplementary Fig. 3). When 3 ng μl−1 cDNA concentration was used, SEP–GluK2 clusters detected by confocal microscopy were selectively localized at TEs and were barely detectable in the soma or in DDs (number of clusters per μm length: 3 ng μl−1: soma, 0.227 ± 0.012; TE, 0.19 ± 0.045; DD, 0.03 ± 0.036; Fig. 5a,b). In contrast, at high cDNA concentration (80 ng μl−1), SEP–GluK2 clusters were abundant in both the soma and DDs, although the highest levels were again detected at TEs (number of clusters per μm length: 80 ng μl−1: soma, 0.132 ± 0.036; TE, 0.436 ± 0.097; DD, 0.215 ± 0.023; Fig. 5a,b). Overall, these morphological data corroborate the functional subcellular distribution of KARs and confirm that aberrant ectopic KARs are expressed when a high concentration of cDNA is used.

**Figure 3 | Block of KAR currents by the GluK2α/GluK5 antagonist UBP310.** (a) Sample traces illustrating KAR currents evoked by glutamate uncaging on TEs of CA3 PCs in organotypic slice cultures prepared from GluK2+/+ and GluK2−/− not transfected (NT) or transfected with 3 ng μl−1 of GluK2a cDNA or 80 ng μl−1 in the absence or presence of UBP 310 (3 μM). (b) Bar graph summarizing the percentage of inhibition of UBP 310 (3 μM) on the KAR currents evoked by glutamate uncaging. In GluK2+/+ NT: n = 6 cells; 3 ng μl−1: n = 7 cells; 3 ng μl−1: n = 12 cells; 7 mice. Values are presented as mean ± s.e.m. of n experiments. Data were compared using one-way analysis of variance followed by Dunnett’s multiple comparison test (NS, not significant; P > 0.05 and **P < 0.01).
Gluk2–/– GluK2–/– mice, not transfected (NT) or transfected with different amounts of GluK2a cDNA (3 ng μl–1) (top) or 80 ng μl–1 (bottom). SEP–GluK2 clusters. Representative confocal z-stack projections are shown for each condition. Insets represent characteristic CA3 TEs or DDs. Arrows show the co-localized SEP-K2 (80 ng μl–1) and anti-GFP nanobody signals that correspond to SEP–GluK2 clusters. (d,e) Sample traces of mf-CA3 KAR-EPSCs shown in a and scaled at the peak illustrating the lack of difference in the decay kinetics for various conditions. (e) Quantification of the decay rate (tau weighted) of KAR-EPSCs from cells transfected with different amount of cDNA encoding for GluK2a. In all panels, values are presented as mean ± s.e.m. of n experiments. In c, data were compared using one-way analysis of variance followed by Dunnett’s multiple comparison test, except for A/C experiments that were compared with unpaired t-test (NS, not significant; *P < 0.05 and ****P < 0.0001).

Figure 5 | Subcellular distribution of SEP–GluK2a in CA3 PCs. (a) Sample images illustrating CA3 neurons electroporated with 3 ng μl–1 (top) or 80 ng μl–1 (bottom) SEP–GluK2 cDNA (green). TdTomato (blue) and live stained for the SEP moiety using anti-GFP nanobody-ATTO647 (red). Representative confocal z-stack projections are shown for each condition. Insets represent characteristic CA3 TEs or DDs. Arrows show the co-localized SEP and anti-GFP nanobody signals that correspond to SEP–GluK2 clusters. (b) Average number of clusters per unit length at the soma (S), TE or DD for each condition (3 ng μl–1: n = 11 cells, 3 different experiments, 3 mice; 80 ng μl–1: n = 10 cells, 3 different experiments, 3 mice). In all panels, values are presented as mean ± s.e.m. of n experiments. Data were compared using unpaired t-test (*P < 0.05 and ****P < 0.0001).

GluK2a C terminus stabilizes KARs at mf-CA3 synapses. The CTD of GluK2 is the target of multiple proteins that modulate the trafficking of KARs to the plasma membrane.6,15,37–39 We studied the role of the CTD of GluK2a in the stabilization of KARs at mf-CA3 synapses by reexpressing different truncated forms of GluK2a subunits in CA3 PCs in organotypic slices of GluK2–/– mice (Fig. 6a). The cDNAs (3 ng μl–1) coding for different GluK2a subunits truncated in the CTD (GluK2aΔ4, GluK2aΔ14; GluK2aΔ29 and GluK2aΔ39) were electroporated in CA3 PCs (Fig. 6a). AMPAR- and KAR-EPSCs were evoked by mf electrical stimulation (Fig. 6b,c). Re-expression of GluK2aΔ4, a GluK2a subunit lacking the PDZ domain-binding motif necessary for the interaction with PSD95, rescued KAR-EPSCs with a relative amplitude comparable to WT (KAR/AMPAR ratio WT: 10.0 ± 1.3%; GluK2aΔ4: 9.7 ± 1.4%), suggesting that this molecular interaction is not critical for the retention of KARs.
to mf-CA3 synapses. Interestingly, re-expression of both GluK2aΔ14 and GluK2aΔ29 strongly reduced KAR-EPSCs (KAR/AMPAR ratio, GluK2aΔ14: 4.8 ± 0.8%; P < 0.01; GluK2aΔ29: 3.4 ± 0.7%; P < 0.001), albeit trafficking of GluK2aΔ14 and GluK2aΔ29 to the plasma membrane is not impaired in dissociated cultured neurons. In addition, the biophysical properties of GluK2a truncated up to the last 29 amino acids do not differ from full-length GluK2a. Hence, the decreased amplitude of KAR-EPSCs may be explained by impaired recruitment or stabilization of mutated KARs at mf-CA3 synapses. Further truncation of the last 39 amino acids (GluK2aΔ39) is known to decrease surface trafficking of GluK2a due to defect in forward trafficking of KARs to the plasma membrane. Accordingly, expression of GluK2aΔ39 (3 ng μl⁻¹) in GluK2−/− mice led to KARs-EPSCs with largely reduced relative amplitude with respect to WT (KAR/AMPAR ratio, WT: 10.0 ± 1.3; GluK2aΔ39: 2.0 ± 0.5; P < 0.001). KAR currents evoked by glutamate uncaging on TE following re-expression of GluK2aΔ14 (3 ng μl⁻¹) and GluK2aΔ29 displayed largely decreased mean amplitudes as compared with WT (mean amplitude, WT: 59.7 ± 12.0 pA; GluK2aΔ14: 15.9 ± 1.8 pA; GluK2aΔ29: 19.3 ± 2.6 pA; P < 0.001; Fig. 6d,e). No detectable extrasynaptic KAR currents were however observed in the soma following re-expression of GluK2aΔ14 or GluK2aΔ29 in GluK2−/− mice (Supplementary Fig. 4). Together, these results confirm that the 14 last amino acids of GluK2a CTD are important for the stabilization of KARs at mf-CA3 synapses.

N-cadherin is involved in synaptic stabilization KARs. GluK2a interacts with N-cadherin/β-catenin complexes through its CTD domain; this interaction involves the 14 last amino acids of the CTD and is necessary for N-cadherin-mediated recruitment and aggregation of membrane KARs in heterologous cells. As truncation of this 14 amino-acid sequence strongly decreased KAR-EPSC amplitude, we tested the role of the N-cadherin/β-catenin complex in the stabilization of KARs at mf-CA3 synapses. For this, we first used a dominant-negative approach in organotypic cultures by overexpressing in CA3 cells an N-cadherin truncated (Fig. 4). Together, these results confirm the involvement of N-cadherin in the stabilization of KARs at mf-CA3 synapses.
Figure 7 | N-cadherin is necessary for the stabilization of synaptic KARs. (a,b) Averaged synaptic currents evoked by mf stimulation (3Hz) in WT CA3 PCs from organotypic slices transfected with GFP or NCad-ΔE (30 ng μl−1) in the absence and in the presence of 25 μM LY303070, to isolate the AMPAR and the KAR component, respectively. Insert: enlargements of isolated KAR EPSCs obtained from experiments presented in (a). GFP-Cre; uncaging sites: S, TE, DD, S, TE, DD; GFP-Cre: soma, 18.9 ± 1.7 pA; DD, 4.7 ± 0.8 pA; Fig. 7e,f). In contrast, the amplitude of AMPAR-mediated currents were not significantly reduced on N-cadherin knockdown with respect to control conditions (mean amplitude, nuclear GFP: soma, 177.5 ± 33.3 pA; TE, 331.2 ± 36.6 pA; DD, 80.2 ± 19.8 pA; GFP-Cre: soma, 122.9 ± 24.8 pA; TE, 264.6 ± 42.7 pA; DD, 61.9 ± 15.4 pA; Fig. 7g,h). We obtained similar results when the knockdown of N-cadherin was induced in vivo by stereotaxic injection of N-Cadherinβ/ΔαI4 mice with a virus (AAV 2.9) coding for GFP-Cre, under the control of a synapsin promoter in the CA3 area of the hippocampus. Acute brain slices were prepared and patch-clamp recordings of CA3 PCs were performed 3–4 weeks after infection (Supplementary Fig. 5). Altogether, these results indicate that the N-cadherin/β-catenin complex is involved in the recruitment and/or stabilization of KARs at mf-CA3 synapses.

Discussion

We show that the strict compartmentalization of KARs at mf-CA3 synapses in CA3 PCs is readily preserved in organotypic slices, providing a much improved model over dissociated cultured neurons, which express functional native KARs, however not synthetically. Similar to that in acute slices, focal glutamate

Ncad-ΔE (mean net amplitude, GFP: 114.1 ± 11.5 pA; Ncad-ΔE: 109.8 ± 15.2 pA; Fig. 7b).

Ncad-ΔE may potentially affect signalling through different sources of KARs and N-cadherin, we deleted endogenous N-cadherin by expression of the Cre recombinase in CA3 PCs from N-cadherin floxed mice (N-Cadherinβ/ΔαI)43 with a plasmid coding for GFP-cre or for nuclear GFP as a control (Fig. 7c,d). We also observed a marked reduction of KAR EPSCs in control conditions (mean amplitude, nuclear GFP: soma, 177.5 ± 33.3 pA; TE, 331.2 ± 36.6 pA; DD, 80.2 ± 19.8 pA; GFP-Cre: soma, 122.9 ± 24.8 pA; TE, 264.6 ± 42.7 pA; DD, 61.9 ± 15.4 pA; Fig. 7e,f). In contrast, the amplitude of AMPAR-mediated currents were not significantly reduced on N-cadherin knockdown with respect to control conditions (mean amplitude, nuclear GFP: soma, 177.5 ± 33.3 pA; TE, 331.2 ± 36.6 pA; DD, 80.2 ± 19.8 pA; GFP-Cre: soma, 122.9 ± 24.8 pA; TE, 264.6 ± 42.7 pA; DD, 61.9 ± 15.4 pA; Fig. 7g,h). We obtained similar results when the knockdown of N-cadherin was induced in vivo by stereotaxic injection of N-Cadherinβ/ΔαI4 mice with a virus (AAV 2.9) coding for GFP-Cre, under the control of a synapsin promoter in the CA3 area of the hippocampus. Acute brain slices were prepared and patch-clamp recordings of CA3 PCs were performed 3–4 weeks after infection (Supplementary Fig. 5). Altogether, these results indicate that the N-cadherin/β-catenin complex is involved in the recruitment and/or stabilization of KARs at mf-CA3 synapses.

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uncaging in CA3 PCs from WT organotypic slices reveals a highly segregated subcellular distribution of functional KARs. KAR currents can only be activated when the uncaging spot is focalized on TEs, but not on the soma, on the proximal dendritic shaft or on distal dendrites and spines. Pathway-specific localization of postsynaptic KARs may be a general feature, which has indeed been reported in pyramidal neurons of the medial entorhinal cortex and at climbing fibre synapses onto Purkinje cells, indicating that confined expression of KARs can be observed in the absence of a morphologically specialized postsynaptic element such as TEs. The spatial resolution of our technique, together with the spacing between PSDs of mf-CA3 synapses (ca. 500 nm), does not allow to exclude the presence of KARs at perisynaptic sites between the various PSDs on TEs. However, functional studies using blockers of glutamate transporters to probe for activation of KARs by glutamate spillover do not favour the presence of KARs at perisynaptic sites. Nonetheless, our experiments reveal a striking difference between the two related iGluR families AMPARs and KARs. Whereas AMPARs are present at both synaptic and extrasynaptic (soma and dendritic shafts) sites in acute and organotypic slices, KARs are exclusively distributed at a particular synaptic input and absent from extrasynaptic sites. Extrasynaptic AMPARs may serve as a pool of receptors ready to be used for LTP of AMPAR-EPSCs relying on surface diffusion. The lack of a pool of extrasynaptic KARs may explain the inability to induce LTP of KAR-EPSCs, except in artificial conditions where AMPARs are absent.

With this model system in hands, we identified molecular sequences of the major KAR subunit GluK2 required for the strict subcellular segregation observed. We transfected CA3 PCs from GluK2+/−/+ slices to re-express the GluK2a subunit and mutants thereof, based on the fact that GluK2 is an essential component of KARs in CA3 PCs. From a methodological standpoint, we found that transfection of a precise amount of GluK2a cDNA, using single-cell electroporation, was necessary for a faithful restoration of the strict subcellular segregation of KARs. With concentrations of cDNA above 10 ng μl−1, re-expression of GluK2a led to the abnormal expression of KARs throughout the somatodendritic compartment of CA3 PCs. Beside the strong methodological warning about the importance of using a semi-quantitative replacement strategy, these results also suggest that the stringent subcellular segregation of native KARs—the exclusion from extrasynaptic sites—depends at least in part on the amount of the GluK2 subunit expressed by CA3 PCs. Hence, in physiological conditions, one can calculate that the amount of GluK2-containing KARs is strongly controlled, either at the transcriptional or posttranscriptional level. Not much is known about transcriptional regulation of KARs. At the posttranscriptional level, SUMOylation of GluK2a leads to the removal of KARs from the postsynaptic membrane, leading to a decrease in KAR-mediated synaptic transmission. In addition, the presence of GluK2 in the plasma membrane can be controlled by its interaction with Parkin, which interacts and ubiquitinates GluK2, and as a consequence regulates GluK2 levels and KAR currents in cultured neurons. It would thus be interesting to map KARs in CA3 PCs in the absence of Parkin, or in conditions of reduced SUMOylation of GluK2.

The large ectopic KAR currents evoked by brief ultraviolet uncaging flashes observed in CA3 PCs transfected with high concentrations of cDNA displayed fast decay kinetics, which are reminiscent of recombinant homomeric GluK2 KARs and are very different from native KARs recorded on TEs. The block of ectopic KAR currents by UBP310, an antagonist of homomeric GluK2/GluK5 receptors but not of homomeric GluK2 (ref. 33), indicates that ectopic KARs are probably homo-tetramers. This suggests that the availability of GluK4 or GluK5 to form heteromers are in controlled and limited amounts in CA3 PCs. Hence, non-controlled overexpression of GluK2 leads to the loss of the strict compartmentalization of KARs in CA3 PCs giving rise to abnormal homomeric GluK2 at extrasynaptic sites.

Re-expression of GluK2a in CA3 PCs of GluK2−/− mice restores expression of KARs at mf-CA3 synapses. Interestingly, the amplitude and kinetics of KAR-EPSCs appear independent of the amount of cDNA transfected, a finding compatible with a synaptic slot hypothesis. Whatever the cDNA concentration, KAR-EPSCs cannot be recorded at A/C synapses. We thus propose that only a limited amount of slots selective for KARs are available at mf-CA3 synapses. Once these slots are occupied, overexpressed KARs invade extrasynaptic sites. The limited slots for KARs correlates with the observation that in physiological conditions, KAR-EPSCs can undergo LTD, but not LTP. LTD may correspond to the diffusion of heteromers of GluK2 and/or GluK4 and GluK5 out of synapses, followed by rapid internalization. GluK4 and GluK5 are certainly essential elements in controlling the synaptic amount of KARs and removing both GluK4 and GluK5 abolishes KAR-EPSCs.

What constitutes a KAR synaptic slot remains an open question. Possibilities include PDZ-domain-containing proteins such as PSD95, PICK or GRIP, although re-expression of a mutated GluK2a subunit lacking the four last amino acids responsible for the interaction with PDZ domain proteins did not affect KAR-EPSCs. In contrast, removal of the last 14 amino acids of GluK2a strongly had an impact on the amplitude of KAR-EPSCs. Interestingly, these last 14 amino acids are essential for the interaction of GluK2-containing KARs with N-cadherin/β-catenin complexes, albeit this interaction is likely to be indirect and other proteins could potentially interact with GluK2 through these amino acids. N-cadherins are synaptic adhesion proteins implicated in synapse formation and maturation. Activation of N-cadherins by ligand-covered latex beads recruits GluK2a to N-cadherin/β-catenin complexes in heterologous cells in a manner, which strictly depends on the last 14 aa of GluK2a. Here we demonstrate that overexpression of a dominant-negative N-cadherin or knocking down N-cadherin in slice cultures or in vivo strongly reduces KAR-EPSCs at mf-CA3 synapses without impacting on basal synaptic properties. This decrease in KAR-EPSCs was not accompanied with a detectable increase in extrasynaptic KARs, suggesting that unstabilized synaptic KARs may undergo rapid endocytosis. Although N-cadherin binds AMPARs through an interaction with the N-terminal extracellular domain of GluA2 (ref. 56), or intracellularly, AMPARs were surprisingly not affected by the removal of N-cadherin in our experimental conditions. This was indicated by the lack of effect of N-cadherin inactivation on the amplitude of evoked AMPAR-EPSCs, mEPSCs and AMPAR currents induced by focal glutamate uncaging. Possibly, AMPARs may be stabilized at the PSD in mature synapses via additional transsynaptic adhesion molecules, such as neuroligins or leucine-rich repeat transmembrane (LRRTM) proteins. Hence, our data indicate that N-cadherins specifically recruit and/or stabilize KARs at mf-CA3 synapses.

Synaptic specificity is probably mediated by a network of synaptic molecules. N-cadherins appear to be one of the partner molecules responsible for the synaptic stabilization of KARs at mf-CA3 synapses. Accordingly, it is interesting to note that N-cadherin labelling is particularly intense in the dendritic shaft of CA3 PCs in the stratum lucidum. Other partners are likely to play a role in the subcellular segregation of KARs at these particular synaptic sites. Synaptic specificity in the brain involves differential expression of type II cadherin genes, such as Cadherin-9, which is expressed in both the DG and CA3 PCs, and appears essential for the specific formation of mf-CA3 synapses.
during development. Cadherin-9 may also play a role in specifying the functional properties of mature mF-CA3 synapses, although this has yet to be explored. An association between N-cadherin and Cadherin-9, possibly assembled as heterodimers, could be essential for the maintenance of specific synaptic features. One can speculate that the loss of Cadherin-9 could similarly impact KAR-EPSCs. Interestingly, a recent report identifies a role for the Clq-like proteins ClqL2 and ClqL3 in the recruitment of KARs at mF-CA3 synapses by direct interaction with the extracellular domain of GluK2 and/or GluK4 subunits. One can speculate that the ClqL2/3 proteins secreted by mfs may regulate the recruitment of KARs and provide input specificity, whereas N-cadherins would stabilize synaptic KARs through indirect interactions with the cytoplasmic domain of GluK2a.

Understanding the molecular mechanisms underlying the synaptic recruitment of KARs at mF-CA3 synapses could also shed light on the pathological process through which aberrant KARs are recruited at recurrent mf onto DG cells in temporal lobe epilepsy. Neutralization of these aberrant KARs markedly attenuated CA3 to CA1 responses in a hippocampal slice model of temporal lobe epilepsy. The molecular mechanisms underlying synaptic segregation of KARs probably bear similarities at both mF-CA3 synapses and recurrent mf synapses onto DG cells.

In conclusion, multiple mechanisms combine to control the specific subcellular localization of KARs at mF-CA3 synapses, including a stringent control of the amount of GluK2 subunit in CA3 PCs, a limited number of postsynaptic slots for KARs and the recruitment/stabilization of KARs by transsynaptic mechanisms which may involve a coordination between secreted ClqL2/3 proteins, cadherin-9 and N-cadherin.

**Methods**

**DNA constructs.** GluK2a WT and GluK2a C-terminal mutant cDNAs used in the present study were described previously and were expressed under the control of a cytomegalovirus (CMV) promoter. DNA plasmids were dissolved at various concentrations in the electroporation solution: GluK2 WT, GluK2 C-terminal mutant and SEP–GluK2 (ref. 62) cDNAs at concentration between 3 and 80 ng μl⁻¹, GFP at 30 ng μl⁻¹ (CMV), tdTomato, 20 ng μl⁻¹ (CMV); NCad-ΔE (33) at 30 ng μl⁻¹ (CMV); GFP–cre at 30 ng μl⁻¹ (CMV) and nuclear GFP 30 ng μl⁻¹ (CMV). In all uncaging experiments, CA3 PCs were co-transfected with GFP or tdTomato to visualize cell morphology. In uncaging experiments, NT refers to cells not transfected with GluK2a (or NCad-ΔE, GFP–cre or nuclear GFP) plasmids, but expressing only GFP or tdTomato.

**Slice culture preparation and transfection.** Organotypic hippocampal slice (300 μm thickness) cultures were prepared from P5 to P7 GluK2−/− and WT FVB mice as described previously and, according to the guidelines of the University of Bordeaux/CNRS Animal Care and Use Committee. Three to 4 days after plating, the medium was replaced and then changed every 2–3 days. After 7 days in vitro, visually identified CA3 PCs were transfected by single-cell electroporation. For this, slices were placed in the microscope chamber in the presence of a small volume of a sterile pre-warmed (37°C) HEPES-based artificial cerebrospinal fluid (ACSF) composed of 145 mM NaCl, 2.5 mM KCl, 2.5 mM NaH2PO4, 26 mM NaHCO3, 2.3 mM CaCl2, 1.3 mM MgCl2, 10 mM glucose and 1 mM sodium pyruvate. One to three cells were recorded per slice. A train of depolarizing currents was adjusted to evoke glutamate uncaging in the presence of MNI glutamate 500 μM (2 ms duration at 0.1 Hz). Glutamate uncaging was performed on a Nikon FN1 microscope equipped with a × 60 lens (numerical aperture 1.00) and a 405 nm Obis laser (Coherent, Germany; 10 mW CW) controlled by a Digitimer DS2A (Digitimer, Welwyn, UK). A membrane access resistance was below 20 MΩ and was monitored during the entire experiment. No series resistance compensation was used. Recordings were made using EPC 9 or EPC10 amplifiers (HEKA Elektronik, Lambrecht/Pfalz, Germany), which were filtered at 0.1–3 kHz, digitalized at 5 kHz and stored on a personal computer for further analysis (IGOR PRO 6.2, WaveMetrics, Lake Oswego, OR). Values are presented as mean ± s.e.m. throughout the manuscript. Images were acquired using the IGOR PRO software through NeuroMatic v2.6 plugin. The decay phase of the currents was fitted with a function of the following form: \( I(t) = \sum A_i \exp(-t/\tau_i) \), where \( A_i \) is the fraction of respective components \( (A_i = 1) \) and \( \tau_i \) is the time constant of the \( i \)th component. A maximum of two exponential components was used. It has been shown that in organotypic slice cultures A/C fast synaptic transmission, unlike mF-CA3, can be in part mediated by P2X receptors activated by ATP. We have observed similar residual currents in our preparation both in the presence of NBQX 25 μM and in GluK2−/− animals (data not shown). For this reason, to quantify KAR-mediated EPSCs at A/C input we have subtracted the residual current after application of LY 30,791, with the residual current after the further application of NBQX (to block AMPARs and KARs).

**Electrophysiology.** Whole-cell patch-clamp experiments were performed at 33 °C from CA3 PC expressing or not expressing GluK2−/− after 2–3 days after transfection. Whole-cell patch-clamp recordings were performed with 3–4 MΩ patch pipettes with a solution containing 140 mM CsCl, 2 mM MgCl2, 4 mM NaCl, 5 mM P-citrate, 3 mM Na2ATP, 0.33 mM GTP, 0.2 mM EGTA and 10 mM HEPES adjust to 300 mOsm l⁻¹ and pH 7.2 with CsOH. In experiments in which mf or A/C inputs were electrically stimulated by an extracellular electrode, slices were perfused with an extracellular solution (ACSFM): composed of 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaHPO4, 26 mM NaHCO3, 2.3 mM CaCl2, 1.3 mM MgCl2, and 25 mM glucose saturated with 5% O2/5% CO2. Mg synaptic responses were evoked by minimal stimulation by placing a patch pipette filled with aCSF in the hilus of the DG. A/C excitatory stimulations were evoked by placing the pipette in the CA1 radiatum above the CA3 PC layer. The distinct nature of the two synaptic inputs was confirmed by recording short-term plasticity properties. These experiments were performed at 30–32 °C in the presence of pharmacological drugs in the aCSF: NBQX 150 mM, DAP 50 mM and bicuculline 10 μM. For experiments with synaptic stimulation, one cell was recorded per slice. KAR currents were expressed as the ratio of peak inward currents (EPC9 or EPC10 amplifiers) in the presence of NBQX 25 μM and bicuculline 10 μM to the current in the absence of both drugs. AMPA receptor responses were recorded after an average latency of 30 ms, while KAR responses were evoked after a latency of 10 ms.

**Immunohistochemistry and SEP–GluK2a.** Live organotypic hippocampal slices in which CA3 PCs were electroporated with tdTomato and SEP–GluK2a were labelled with 200 nM GFP-nanobody coupled to ATTO647N (ATTO-TEC) in a dark chamber for 30–60 min at room temperature in the same aCSF used for uncaging experiments supplemented with 1% globulin-free BSA (Sigma, St Louis, MO). slices were then rinsed and fixed in 4% paraformaldehyde (PFA) containing 20% sucrose for 2 h at 4°C, before rinsing with PBS and mounting in mowiol 4–88 (Sigma). Confocal images were acquired sequentially on a commercial Leica TCS SPE microscope equipped with three laser diodes (488, 561, 647 nm) using a × 63 oil objective and a pinhole opened to one time the Airysk. The number of SEP–GluK2a clusters per micrometer was determined using MetaMorph (Molecular Devices, USA). Briefly, z-projections of the stacks in the 488 (SEP) and 635 (ATTO647N) channels were segmented after background subtraction. Segmented images were over laid and GluK2 clusters were defined as individual spots that were present in both channels and co-localized within defined areas of the neuron ( soma, TEIs and DIs). In Fig. 1 and Supplementary Fig. 1, the confocal images were obtained after fixation of organotypic slices following glutamate uncaging on organotypic slices from WT FVB mice electroporated with a cDNA construct coding for GFP-N1. The slices were fixed with PFA, 30% sucrose for 15 min at room temperature. After blocking with PBS, 10% fetal calf serum for 1 h, we immunostained GFP by incubating the slices with a mouse monoclonal antibody anti-GFP (Roche; dilution 1/200, overnight at 4°C) in PBS containing 0.2% Triton and 5% SVF. After washing three times in PBS, the slices were incubated with a fluorescein-coupled secondary antibody anti-mouse (Life Technologies) in PBS containing 5% SVF and 1% Triton for 1 h, with PBS washes for 1 h at room temperature. Finally, the slices were washed three times at room temperature with PBS and oversprayed in Vectashield medium (Vector).
Confocal laser-scanning fluorescence microscopy and image analysis. Confocal image stacks of PFA-fixed organotypic slices were acquired with a Leica DM6000B TCS QSP SR-X while laser-scanning microscope using the objective Leica HCX PL Apo CS × 100 oil numerical aperture 1.4. Images were processed with ImageJ 1.49d software (Wayne Rasband, National Institutes of Health, USA). Illustrations correspond to two-dimensional maximal projections of three-dimensional acquired confocal stacks smoothed by the median filter.

Drugs. All drugs were obtained from Tocris Cookson (Bristol, UK), Sigma or Ascent Scientific (Bristol, UK).

Data availability. The data that support the findings of this study are available from the corresponding author on request.

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Acknowledgements
This study was supported by the Centre National de la Recherche Scientifique, the Conseil Régional d’Aquitaine, the European Commission (EIF Fellowship awarded to MC), the Agence Nationale de la Recherche (contract MossyGlu), the labex BRAIN and the Fondation pour la Recherche Médicale (FDT20140931189 to S.F.). We acknowledge Françoise Coussen for fruitful initial discussions and help with the GluK2a plasmids. We thank Séverine Deforges for producing the plasmid DNA; Elisabeth Normand, Audrey Lacquemant and Amandine Gautier for taking care of the mice; Adam Gorlewicz for help with immunocytochemistry and for discussion on Cadherins; Federico Trigo for help in setting up the uncaging experiments; and Matthieu Sainlos for the help on the production of anti-GFP nanobody. Cellular imaging was in part performed at the Bordeaux Imaging Center, a service unit of the CNRS-INSERM and Bordeaux University, member of the National Infrastructure France BioImaging.

Author contributions
S.F. performed and analysed slice electrophysiology experiments in combination with pharmacological and molecular replacement approaches with the help of M.C. M.C. initiated the project and developed single-cell electroporation of KAR cDNAs in hippocampal slices. I.C. performed image acquisition and analysis of SEP–GluK2a experiments. O.T. helped design the imaging experiments and contributed to discussion. V.L. helped S.F. to perform image acquisition and analysis. C.M. supervised the project and wrote the manuscript together with S.F. and M.C.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Fievré, S. et al. Molecular determinants for the strictly compartmentalized expression of kainate receptors in CA3 pyramidal cells. Nat. Commun. 7:12738 doi: 10.1038/ncomms12738 (2016).

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