The deletion of GluK2 alters cholinergic control of neuronal excitability

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ABSTRACT

Kainate receptors (KARs) are key regulators of synaptic circuits by acting at pre- and postsynaptic sites through either ionotropic or metabotropic actions. KARs can be activated by kainate, a potent neurotoxin, which induces acute convulsions. Here we report that the acute convulsive effect of kainate mostly depends on GluK2/GluK5 containing KARs. In contrast, the acute convulsive activity of pilocarpine and pentylentetrazol is not alleviated in the absence of KARs. Unexpectedly, the genetic inactivation of GluK2 rather confers increased susceptibility to acute pilocarpine-induced seizures. The mechanism involves an enhanced excitability of GluK2^{-/-} CA3 pyramidal cells compared to controls upon pilocarpine application. Finally, we uncover that the absence of GluK2 increases pilocarpine modulation of Kv7/M currents. Taken together our findings reveal that GluK2-containing KARs can control the excitability of hippocampal circuits through interaction with the neuromodulatory cholinergic system.

INTRODUCTION

Kainate receptors (KARs), which belong to the family of ionotropic glutamate receptors alongside AMPA and NMDA receptors, are widely expressed in the brain (Contractor et al. 2011). Five KAR subunits (GluK1, GluK2, GluK3, GluK4 and GluK5) co-assemble in various combinations to form functional homomeric and heteromeric receptors (Contractor et al. 2011; Lerma and Marques 2013). KARs regulate the activity of neuronal circuits by a variety of mechanisms which depend on subunit composition, cell-specific expression, pre- or postsynaptic localization, and signaling pathways (Mulle and Crépel 2021; Lerma and Marques 2013). These diverse roles modulate synaptic transmission and the balance between excitation and inhibition in the hippocampus, hence the overall activity of synaptic circuits (Carta et al. 2014).

KARs were initially defined by their high affinity for kainate (Bettler and Mulle 1995), a potent neurotoxin. Systemic or intracerebral administration of kainate, an agonist of AMPARs and KARs, acutely induces behavioral and electrophysiological seizures reminiscent of those found in patients with temporal lobe epilepsy (TLE) (for review see Traynelis et al. 2010; Crépel and Mulle 2015). In TLE, the most common form of human epilepsy, recurrent partial seizures originate from mesial structures such as the hippocampus and propagate to the other limbic constituents, finally giving rise to general convulsions (*status epilepticus*) (Bertram 2009). Although the acute seizures triggered by kainate may have been fruitful for the understanding of epileptogenesis and the discovery of antiepileptic drugs, these do not closely match the complex features of chronic seizures in TLE (Rogawski 2006). GluK2-containing KARs, which are widely expressed in the brain represent the main target for kainate-induced acute seizures (Mulle et al. 1998a). The role of GluK1 in epileptogenesis is less clear. There is evidence that GluK1 agonists act as anticonvulsants by activation of interneurons (Khalilov et al. 2002), and conversely, evidence that GluK1 antagonists may prevent seizures in a pilocarpine model of TLE. Recently, the use of GluK1^{-/-} mice has clearly indicated a role for this subunit in acute seizures induced by systemic administration of the potent GluK1 agonist ATPA, but not of kainate or pentylentetrazol (PTZ) (Fritsch et al. 2014). To clarify the role of KAR subunits in the generation of acute seizures, we have systematically examined the propensity to acute seizure generation of kainate in knock-out mice for GluK1, GluK2, GluK3 and GluK5.

Other pharmacological models have utilized systemic administration of convulsive agents such as pilocarpine, a cholinergic agonist, and PTZ, a GABAergic antagonist to induce acute seizures reminiscent of *status epilepticus* in TLE in the rodent (Löscher et al. 1991; Lévesque et al. 2016). Here we asked whether KARs were also involved in the convulsive action of PTZ and pilocarpine. The analysis of KAR subunit knock-out mice indicates that acute behavioral seizures do not depend on KARs in these models. Our work leads to the unexpected finding

66 that GluK2^{-/-} mice show enhanced susceptibility to pilocarpine-induced acute seizures. We
67 then performed electrophysiological experiments to better understand the mechanisms linking
68 KARs and increased neuronal excitability induced by bath application of pilocarpine in
69 hippocampal slices. We provide further evidence that the deletion of GluK2 alters the
70 cholinergic control of excitability through inhibition of Kv7/M-currents.

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MATERIALS AND METHODS

Animals. Experimental procedures followed standard international regulation (European Commission Directive 86/609/EEC), as well as the recommendations of the CNRS ethics committee, and the French Ministry of Agriculture and Forestry concerning animal care. Wild-type mice and mutant littermates were derived from heterozygote crossings of GluK1^{-/-} (Mulle et al. 2000), GluK2^{-/-} (Mulle et al. 1998a), GluK3^{-/-} (Pinheiro et al. 2007), GluK5^{-/-} (Contractor et al. 2003) and Thy1-GFP M line mice (Jackson Laboratories). GluK2^{-/-} mice were backcrossed on a C57BL6 and on a FVB background. The GluK1^{-/-}, GluK3^{-/-}, and GluK5^{-/-} mice were backcrossed on a C57BL6 background. Thy1-GFP^{+/-} were backcrossed on C57BL6 background. GluK2-floxed mice were generated in the lab of Anis Contractor (Marshall et al. 2018).

Viral gene transfer and stereotaxic delivery. Mice (P22-P30) were anesthetized by isoflurane inhalation and systemically injected with buprenorphine (0.2 mg/kg) to prevent post-surgery pain. 200 nl of viral solution (1x 10E8 particles) was injected using a micropump and syringe (Nanofil WPI) at the rate of 100 nl/min in the CA3 (Y: 2.2 mm from lambda; X: ± 2.5 mm from sagittal suture; Z: 2.35 mm from the skull) to infect GCs. The spreading of viruses allowed the infection of a large part of the hippocampus (radius of roughly 1 mm) to produce numerous slices for electrophysiology. Experiments were performed at least 2 weeks post-injection.

Kainate injection. Adult male mice (8-12 weeks old) were intraperitoneally injected with kainic acid dissolved in distilled water (dH₂O) (20 mg/kg) (Tocris). After kainate administration, each animal was assigned to one of the following stages depending on the severity of behavioral symptoms. The response scale was modified from the seizure scale developed by Racine (Racine 1972) for the pharmacological kindling model. Stage 0: no observable effect; Stage 1: immobility and rigidity, stretching of the body, tail stiffness, bulging eyes; Stage 2: repetitive shaking of the head, circular movements; Stage 3: falling, non-continuous tonic-clonic seizures with periods of total immobility; Stage 4: continuous tonic-clonic seizures, loss of posture control; Stage 5: death.

Pilocarpine injection. Adult male mice (8-12 week old) were injected intraperitoneally with a single dose of methylscopolamine nitrate dissolved in dH₂O (1 mg/kg) (Sigma-Aldrich) (Turski et al. 1984) to block the peripheral effects of pilocarpine. Twenty minutes after the methylscopolamine administration, a single dose of pilocarpine hydrochloride dissolved in dH₂O (Sigma-Aldrich) was injected intraperitoneally (200 mg/kg, 265 mg/kg 300 mg/kg or 320 mg/kg, depending on the genetic background of the animal and type of the experiment). Animals were observed continuously for 3 hours after pilocarpine administration. Typically within 45 minutes after pilocarpine administration the animals experienced different symptoms of convulsions that were classified using a staging system adopted and modified from the

Racine scale (Racine 1972). Stage 0: no observable effect; Stage 1: immobility and rigidity, repetitive shaking of the head; Stage 2: bilateral clonus of forelimbs, stretching of the body, salivation, chewing; Stage 3: non-continuous tonic-clonic seizures with periods of total immobility; Stage 4: continuous tonic-clonic seizures, loss of posture control; Stage 5: death. Time to the beginning of status epilepticus was measured, as well as the number of fatalities. The onset of status epilepticus was defined as the occurrence of continuous tonic-clonic activity (stage 4).

PTZ injection. Seizures were induced in 8-12 weeks old male mice by the intraperitoneal injection of 45 or 55 mg/kg of PTZ dissolved in dH₂O (Tocris) (Löscher et al. 1991). Within 15 minutes after drug administration animals experienced varied convulsive symptoms. Depending on the symptoms, each of the animals was classified to one of the following stages that were adopted and modified from the Racine scale (Racine 1972): Stage 0: no observable effect; Stage 1: reduced motor activity; Stage 2: partial clonic activity affecting the face, head, vibrissae and forelimbs, jerks of the head and forelimbs, vocalization; Stage 3: whole body clonus without loss of upright posture; Stage 4: whole body clonus with loss of upright posture; Stage 5: whole body clonus with complete loss of motoric control, wild running and jumping; Stage 6 maximal tonic-clonic seizure with tonic extension of hind limbs; Stage 7: death.

Preparation of hippocampal brain slices. For electrophysiology recordings parasagittal brain slices (350 μ m thick) were obtained from 19-24 days old male mice by cutting in an aqueous solution (dH₂O) containing the following (in mM): 80 NaCl, 2.5 KCl, 25 NaHCO₃, 1.5 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂, 10 glucose, and 75 sucrose and equilibrated with 95% O₂ and 5%CO₂. The slices were then incubated at 33°C for 30 min and subsequently stored at room temperature in an aqueous (dH₂O) solution composed of (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 1 MgCl₂, 11 glucose (equilibrated with 95% O₂ and 5%CO₂).

Fluoro-Jade staining. Male mice (8-12 weeks old) were intraperitoneally injected with pilocarpine dissolved in dH₂O (200 mg/kg or 265 mg/kg) as described above, and 24h after pilocarpine administration, the mice were subjected to the following Fluoro-Jade (Millipore) staining protocol. First, mice were perfused transcardially with 4% PFA solution in 0.01M PBS and then the brains were dissected and cut with a vibratome (40 μ m-thick slices). Sections were mounted onto gelatin-coated slides (Menzel-Gläser) and allowed to dry at room temperature (RT). Slides were then placed into a staining rack and immersed in 80% EtOH (in dH₂O) with 1% NaOH (in dH₂O) for 5 minutes; into 70% EtOH (in dH₂O) for 2 minutes; into distilled water (dH₂O) for 1 minute; into a solution of 0.06% potassium permanganate (in dH₂O) for 15 min; dH₂O for 2 min. In the dark, slides were then immersed in a 0.001% Fluoro-Jade (in dH₂O) staining solution for 30 min; rinsed 3x in dH₂O for 1 min; and finally dried at 50°C. Dried slides were at the end rinsed with 100 % xylene for 2 min to clear, immersed in Eukitt (Sigma-Aldrich) mounting medium and coverslipped.

Whole-cell patch clamp recordings in hippocampal brain slices. Borosilicate pipettes (Harvard apparatus) were pulled with a micropipette puller (P97, Sutter Instruments, Novato, CA). Whole-cell current clamp recordings (3–4.5 M Ω electrodes filled with aqueous (dH₂O) intracellular solution (in mM): 140 K-methanesulphonate, 2 MgCl₂, 10 HEPES, 0.3 EGTA, 2 MgATP, 0.3 NaGTP, pH 7.3), and whole-cell voltage clamp recordings (3–4.5 M Ω electrodes filled with an aqueous (in dH₂O) solution containing (in mM): KCl 140, Hepes 10, EGTA 1, MgATP 2, MgCl₂ 2, pH 7.3) were made from hippocampal CA3 pyramidal cells. The osmolality of the internal solutions was 280–290 mOsm/l. Cells were visualized with infrared video-microscopy. Experiments were performed with an extracellular solution near physiological temperature (33°C). No continuous current was injected to keep V_m around the same value during continuous current-clamp recordings. To have homogenous and comparable data sets, cells were rejected from the analysis if the initial V_m value was below -60 mV or above -70 mV. To investigate the action potential firing patterns of neurons, 200 ms depolarizing current steps from 0 pA to 100 pA in increments of 10 pA were applied. The number of action potentials released at each current step was plotted against the amplitude of the current applied. The resting membrane potential (RMP) was averaged over the first 10 s of the 30-minute continuous current clamp recording, or over a 100 ms baseline preceding hyperpolarizing current steps.

Spike after potentials i.e., after-hyperpolarizations (AHPs) and after-depolarizations (ADPs) were measured as the difference (ΔV_m) between the membrane potentials 100 ms before AP (initial V_m: iV_m) and either +10 ms or +100 ms after the peak of the AP. The ΔV_m , analyzed based on spontaneous APs (Fig. 6A) was correlated with the RMP measured 10 ms prior to AP generation (initial V_m). The ΔV_m values were either binned (2 mV bin) according to the initial V_m value, and averaged within a bin, or average cell by cell independent of initial V_m (Fig. 6E, F). To compare input resistance over different sets of data, the steady-state voltage responses were plotted against the amplitude of injected current and the slope of the linear fit of this relationship was characterized by 'R²' (coefficient of determination) and 'a' (linear regression coefficient). To record M-currents (I_M), NaH₂PO₄ was omitted from the extracellular solution. To minimize contamination with other voltage-dependent conductances, I_M was recorded in the presence of TTX (1 μ M), ZD 7288 (20 μ M) and CdCl₂ (500 μ M) to block Na⁺, I_h, Ca²⁺ and Ca²⁺-dependent K⁺ currents, respectively. Xe991 (50 μ M, dissolved in dH₂O) was present in some experiments in order to block Kv7.2/7.3 channels. Capacitance was continuously monitored by applying 10 mV pulses from a holding potential of -70 mV. To minimize voltage errors in voltage-clamp recordings, this was usually compensated by 75–80%. If series resistance changed >20% during the experiments, the data were discarded. Measurements of membrane potentials were not corrected for liquid junction potential error. For I_M deactivation a series of 10 voltage commands (5 mV increment) from the holding

potential of -20 mV was applied. The M-current downregulation was measured as the difference between the initial (<10 ms) and sustained (>100 ms) current of the current trace in control conditions minus the difference calculated alike upon XE991 or pilocarpine treatment.

Immunofluorescence. Fluorescent immunohistochemistry was performed in 50- μ m-thick, free-floating cryosections of perfusion-fixed (4% PFA in 0.01M PBS) brains which were obtained from 8-12 weeks old male mice. The brain sections were subjected to 30-minute incubation with 5% normal donkey serum (NDS) (Jackson Laboratories) and 0.2% Triton X-100 (Sigma) dissolved in dH₂O at RT, followed by overnight incubation with primary antibody diluted in aqueous solutions (in dH₂O) of 5% NDS with 0.2% Triton at 4°C. The detection of immunoreactions was performed using species-specific secondary antibodies directly conjugated to fluorophores (two-hour incubation at RT). Secondary antibodies were diluted in 5% NDS (in dH₂O) with 0.5% Triton (in dH₂O). After incubation with secondary antibodies samples were rinsed with 0.01M PBS, dried on coverslips, immersed in mounting medium and coverslipped. The following primary antibodies and their dilutions were used: rabbit anti-GluK2/K3 (clone NL9, Labome, 04-921) (1:100) and rabbit anti KCNQ2 (Invitrogen, PA1-929) (1:100). The following secondary antibodies were used: donkey anti-rabbit (1:200) conjugated to Alexa555 (Invitrogen).

Data acquisition and analysis. Whole-cell patch clamp recordings were made using an EPC 9 or EPC 10 amplifier (HEKA Elektronik), data were filtered at 3 kHz with a Bessel filter, and digitized at 10 kHz. Off-line data analysis was performed using IGOR PRO (WaveMetrics), and Matlab (MathWorks). Data were acquired with pClamp (Molecular Devices), digitized at 10 kHz, filtered at 3 kHz, and analyzed off-line with built in Clampfit (Molecular Devices) functions. All images were acquired with Leica DM5000 fluorescent microscope (Leica), or NanoZoomer scanner (Hamamatsu). For final inspection, the images were processed using Corel Package as well as standard functions, of the ImageJ program.

For the calculation of the number of Fluoro-Jade positive neurons, stacks of z- planes were maximum-projected with ImageJ (National Institutes of Health, Bethesda, Maryland, USA), and single plane images were subjected to the analysis with the Matlab (MathWorks) script. The algorithm used in the script contained the following steps: automatic threshold-based binaryzation, distance transform, and watershed-based segmentation of the objects, erosion/dilation operation for removal of the isolated pixels, and finally object labelling. Regional fluorescence intensities of KAR and Kv7.2 immunoreactivities were calculated in maximum projected 8-bit images with ImageJ built-in functions. 3D rendering of processed stacks was done using 3DViewer plugin of ImageJ software. 3D models presenting colocalization of Kv7.2 immunoreactivity with giant mossy fiber boutons and thorny excrescence were created using Blender software (Blender foundation).

MS Excel (Microsoft), Matlab (Math Works) and Prism (GraphPad Software Inc.) were used for data management and statistics. To test whether the data follow standard normal distribution we used one-sample Kolmogorov-Smirnov test. To test the null hypothesis that data sets are from populations with the same distribution we used two-sample Kolmogorov-Smirnov test (KS). Either parametric t-test or nonparametric Mann–Whitney U test (MW) were performed to test the null hypothesis that mean values may come from the two data sets with the same distribution. The two-way ANOVA was used to compare the mean differences between the two populations of neurons when analyzing excitability (the number of spikes and respective current step were used as group factors) and when comparing the mean difference between the two populations of neurons when analyzing spike after potentials relationship to membrane potential prior to the peak of the spike. Values are given as means $\pm$ S.E.M. $p < 0.05$ was considered statistically significant. Asterisk notation was used in order to flag the levels of significance ($p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$). For preparation of figures images were processed with Corel Package (Corel Corporation) and MS PowerPoint (Microsoft).

RESULTS

The convulsive effects of kainate depend on specific KAR subunits

In the hippocampus, activation of GluK2-containing KARs with low concentrations of kainate acutely enhances the excitability of CA3 pyramidal neurons leading to the generation and propagation of seizures (Mulle et al. 1998a). We first examined which KAR subunits account for kainate-induced convulsive activity by exploring the sensitivity of mouse mutants for GluK1, GluK2, GluK3 and GluK5. The insensitivity of GluK2^{-/-} mice to kainate was previously reported using mice with a mixed C57Bl6/129Sv background (Mulle et al. 1998a). Here, GluK2^{-/-} mice were backcrossed on two different backgrounds for more than 10 generations (C57Bl6 and FVB) to test whether the insensitivity of GluK2^{-/-} mice to kainate depends on the genetic background. Mice (n=6-10) were injected intraperitoneally with kainate (20 mg/kg). The acute behavioral manifestation of seizure was classified following a modification of the seizure scale developed by Racine (Racine 1972) ranging from 0 when no observable effect was noted, to 5 when the seizure was lethal. We observed a strong resistance to kainate administration in GluK2^{-/-} and GluK5^{-/-} mice as indicated by the plots of the distribution of responses (Fig. 1A). Moreover, the effect of kainate did not depend on the genetic background since GluK2^{-/-} mice on both the FVB and C57Bl6 backgrounds were similarly resistant to the proconvulsant (Fig. 1A). Moreover, kainate administration in GluK1^{-/-} and GluK3^{-/-} animals induced acute seizures to an extent which was not significantly different from control mice, indicating that these two subunits do not participate in kainate-induced acute seizures (Fig. 1A).

GluK2^{-/-} mice show increased susceptibility to pilocarpine-induced acute seizures

The fact that kainate acts through high affinity KARs to generate kainate-induced seizures is however expected. Whether KARs participate in other models of acute seizures in which KARs are not directly targeted by the convulsant has however not been fully characterized. We evaluated the sensitivity of the mutant mice in two common models of acute seizures in rodents, following injection of pilocarpine, a cholinergic muscarinic receptor agonist (Turski et al. 1984) and PTZ, a GABA_A receptor antagonist (Squires et al. 1984). We performed intraperitoneal injections of pilocarpine (265 mg/kg for the FVB background and 320 mg/kg for the C57Bl6 background) and PTZ (45 mg/kg for the FVB background and 55 mg/kg for the C57Bl6 background) and classified the severity of the acute behavioral responses to the convulsants. The necessity to use two different doses of both drugs resulted from the differential sensitivity of mice with different backgrounds (Löscher et al. 2017). The doses of pilocarpine and PTZ suitable for an optimal effect in C57Bl6-based strains were too high in the case of FVB mice, causing intense seizures and high mortality. In order to obtain conclusive data we reduced the doses of convulsants as indicated. We did not find any significant difference in the sensitivity of GluK1^{-/-}, GluK3^{-/-}, and GluK5^{-/-} mice to pilocarpine (n=6-16) (Fig.

1B). Unexpectedly, GluK2^{-/-} mice were more prone to the convulsant effect of pilocarpine than the respective littermate control mice (Fig. 1B). Moreover, we found that the increased susceptibility of GluK2^{-/-} was selective for pilocarpine. Indeed, PTZ-treated GluK2^{-/-} (n=6-12) did not demonstrate any significant difference in their response to the convulsant when compared to control mice on both FVB and C57Bl6 backgrounds. Genetic deletion of GluK1, GluK3 or GluK5 did not alter the severity of PTZ-induced seizures (Fig. 1C) when compared to littermate controls (n=7).

To further investigate the increased sensitivity of GluK2^{-/-} mice to pilocarpine, we monitored the behavior of GluK2^{+/+} and GluK2^{-/-} FVB mice during 120 minutes following injection of pilocarpine (265 mg/kg intraperitoneally). Mice which developed at least stage 3 seizures were further analyzed (n=9-10). Typically, 2-3 very short episodes (several seconds) of tonic-clonic seizures (stage 3) were observed, eventually giving way to continuous limbic motor seizures with intense salivation, rearing, clonus, and falling (stage 4 -*status epilepticus*) (Fig. 2A). GluK2^{-/-} mice experienced the first short episode of tonic-clonic seizure much earlier than GluK2^{+/+} mice (12.5±2 min for GluK2^{-/-}, and 19±2 min for GluK2^{+/+}, p<0.05) (Fig. 2B1). Similarly, GluK2^{-/-} mice developed *status epilepticus* much earlier than GluK2^{+/+} mice (18±4 min for GluK2^{-/-}, and 26±2 min for GluK2^{+/+}, p<0.05) (Fig. 2B1). The fraction of GluK2^{-/-} mice that survived and recovered after *status epilepticus* was lower (10%) than for GluK2^{+/+} mice (70%) (Fig. 2B2). Importantly, a lower dose of pilocarpine (200 mg/kg) which did not induce any observable stage 3 or 4 seizures in control mice, was efficient in triggering epileptiform convulsions in GluK2^{-/-} mice (26±6 min to the first clonic-tonic episode, and 35±5 min to *status epilepticus*) (Fig. 2B1). Hence, in the pilocarpine model of epilepsy GluK2 KARs appear to play a paradoxical protective role, since its absence renders mice more susceptible to seizure induction.

Excessive activation of neurons through ionotropic glutamate receptors, including KARs, can cause generalized neuronal damage (excitotoxicity) *in vitro* in primary cultures, as well as *in vivo* (Vincent and Mulle 2009). CA3 pyramidal neurons and hilar interneurons are amongst the most vulnerable to network hyperactivity and readily degenerate following overexcitation (Wang et al. 2008). We tested whether the enhanced susceptibility of GluK2^{-/-} FVB mice to pilocarpine was reflected in excitotoxic lesions in the hippocampus following pilocarpine administration. Brain sections from both control (n=12) and GluK2^{-/-} (n=12) mice were stained with Fluoro-Jade (a fluorescein-derived fluorochrome which selectively stains damaged neurons) 24h after pilocarpine administration. We observed that pilocarpine (200 mg/kg) induced neuronal death in the hippocampus of GluK2^{-/-} mice as indicated by the numerous Fluoro-Jade positive cells in different hippocampal subregions (Fig. 2C). In contrast, Fluoro-Jade staining was hardly detectable in the same regions in GluK2^{+/+} hippocampal sections (density of Fluoro-Jade positive cells per mm³ in hilus: 6646.4±1420 for GluK2^{-/-} and 530.5±202 for GluK2^{+/+}, p<0,001; in DG: 2834.5±671 for GluK2^{-/-} and 645±25 for GluK2^{+/+}, p<0,001; in

CA3: 3391 ± 1152 for GluK2^{-/-} and 74 ± 42 for GluK2^{+/+}, $p < 0.001$; in CA2: 4681 ± 1725 for GluK2^{-/-} and 67 ± 38.5 for GluK2^{+/+}; in CA1: 3785 ± 925 for GluK2^{+/+} and 113 ± 30 for GluK2^{+/+}, $p < 0.001$ (graph in Fig. 2C). In convergence with behavioral observations, this demonstrates that the effect of pilocarpine is enhanced in GluK2^{-/-} mice.

Enhanced excitation of CA3 pyramidal neurons by pilocarpine in GluK2^{-/-} mice

Considering the important role of GluK2-containing KARs in regulating firing, excitability, and synaptic properties of CA3 pyramidal neurons, we postulated that the increased susceptibility to pilocarpine-induced status epilepticus in GluK2^{-/-} mice may be related to an altered response of CA3 pyramidal neurons to pilocarpine. To explore this possibility we performed current-clamp recordings from CA3 pyramidal cells in control and GluK2^{-/-} brain slices from FVB mice and examined the effects of pilocarpine application on membrane potential and neuronal activity. In control slices ($n=5$), addition of pilocarpine ($30 \mu\text{M}$) resulted in the depolarization of CA3 pyramidal cells (from -67 ± 1 mV to -62 ± 2 mV 20 minutes following pilocarpine application in the bath, $p < 0.001$) (Fig. S1). Addition of atropine suppressed the depolarization induced by pilocarpine confirming that it was mediated by muscarinic acetylcholine receptors (data not shown). To compare the effect of pilocarpine on depolarization in GluK2^{+/+} and GluK2^{-/-} CA3 neurons, the concentration of pilocarpine was reduced from $30 \mu\text{M}$ to $10 \mu\text{M}$ due to a large depolarization and quick deterioration of membrane properties in GluK2^{-/-} neurons upon $30 \mu\text{M}$ pilocarpine treatment. Addition of $10 \mu\text{M}$ pilocarpine caused a statistically significant depolarization of GluK2^{-/-} CA3 neurons ($n=10$) but the membrane potential change at this pilocarpine concentration was not significant in GluK2^{+/+} CA3 neurons ($n=10$) (from -68 ± 1 mV to -65 ± 1 mV for GluK2^{+/+} and from -68 ± 1 mV to -58 ± 1 mV for GluK2^{-/-}, $p < 0.001$) (Fig. 3A) and a robust firing of the CA3 cells. In an effort to investigate whether this phenomenon was cell autonomous, we invalidated GluK2 gene specifically in CA3 cells by viral delivery of the Cre recombinase to CA3 region of GluK2-floxed (GluK2^{fl}) mice (CA3-cKO). First, we isolated the synaptic currents mediated by KARs in CA3 cells by stimulating mossy fibers at 3 Hz under condition where AMPAR-mediated currents are inhibited by LY303070 (Fig. 3B). We observed a total abolition of KAR-mediated currents in CA3-cKO cells (Cre-positive GFP reporting) compared to controls (GFP positive) cells (Fig. 3C) indicating the loss of GluK2 from Cre expressing CA3 pyramidal cells. We then compared responses of CA3 pyramidal cells to hyperpolarizing and depolarizing current steps before (no pilocarpine in the bath) and 20 minutes after pilocarpine ($10 \mu\text{M}$) addition (Fig. 3D). We observed that application of pilocarpine resulted in a higher firing rate in CA3-cKO cells ($n=10$) in comparison to control ($n=10$) CA3 pyramidal cells (12 ± 10 and 27 ± 23 Hz at 100 pA for control and CA3-cKO cells respectively, $p < 0.001$) (Fig. 3E). The hyperpolarization induced by steps of negative current was significantly larger upon pilocarpine application (Fig. 3F). This observation is consistent

with the well-known modulation of cationic conductance by M1 muscarinic receptor activated by pilocarpine. Remarkably, whereas the levels of hyperpolarization were not statistically different between control and CA3-cKO pyramidal cells in control condition, a robust difference appears between the two genotypes upon pilocarpine application (-35 and -68 mV at -100 pA for GluK2^{+/+} and GluK2^{-/-} respectively, $p < 0.001$) (Fig. 3F). Accordingly, the input resistance was increased by application of pilocarpine in both genotypes (Fig. 3G) but the effect was stronger in CA3-cKO cells compared to control CA3 pyramidal cells (343 and 669 MOhm in GluK2^{+/+} and GluK2^{-/-} respectively). This increased membrane resistance likely participates in the increased excitability of CA3 pyramidal cells because the firing rate induced by depolarizing current steps in the presence of pilocarpine was higher in CA3-cKO cells even when the initial membrane voltage was held at -70 mV in both genotypes (Fig. S2). Taken together our data indicate that pilocarpine induces a stronger effect on membrane potential and input resistance in CA3 pyramidal cells in the absence of GluK2 containing KARs. This uncovers a functional interaction between membrane conductance modulation by muscarinic receptors and KARs, which regulates the excitability of CA3 pyramidal cells.

The convulsive action of pilocarpine likely depends on the activation of M1 muscarinic receptor-dependent pathways (Hamilton et al. 1997). Therefore, we tested whether the observed difference in depolarization of CA3 pyramidal cells in response to pilocarpine between genotypes was directly dependent on cholinergic signaling pathways or whether this was an indirect consequence of a general change in the excitability of CA3 pyramidal cells. We recorded the activity of CA3 pyramidal cells in GluK2^{+/+} and GluK2^{-/-} brain slices from FVB mice and tested the effects of gradually increasing K⁺ concentration in the ACSF (from 2.5 mM to 7.5 mM). As expected, increased K⁺ concentration progressively led to depolarization of CA3 pyramidal cells in GluK2^{+/+} (n=10) and GluK2^{-/-} (n=11) slices (Fig. S3). There was no difference in the membrane potential change between the genotypes 20 minutes after ACSF exchange (from -63 mV $\pm$ 1.0 mV to -53 $\pm$ 1.0 mV for GluK2^{+/+} and from -65 $\pm$ 1.0 mV to -53 $\pm$ 1.0 mV for GluK2^{-/-}) (Fig. S3). Moreover, we did not note any difference in the number of action potentials generated by a depolarizing pulse in the presence of 7.5 mM K⁺ (2.6 $\pm$ 0.5 spikes/200 ms at 100 pA for GluK2^{+/+} (n=10) and 3.0 $\pm$ 1 spikes/200 ms at 100 pA step for GluK2^{-/-} (n=11)) (Fig. 4A, traces and top graphs). The I/V relation during hyperpolarizing current steps application was linear and no significant difference was observed in average input resistance of recorded GluK2^{-/-} (n=11) and GluK2^{+/+} (n=10) neurons in control conditions ($R^2 = 0.991 \pm 0.0043$, $a = 340 \pm 22$ for GluK2^{+/+} and $R^2 = 0.996 \pm 0.0043$, $a = 303 \pm 22$ for GluK2^{-/-}) and when high K⁺ concentration was present in the ACSF ($R^2 = 0.979 \pm 0.0101$, $a = 223 \pm 13$ for GluK2^{+/+} and $R^2 = 0.995 \pm 0.0009$, $a = 259 \pm 27$ for GluK2^{-/-}) (Fig. 4A, bottom graphs).

Involvement of GluK2-containing KARs in the modulation of M-currents

It has been shown that neuronal excitability and intrinsic bursting in hippocampal pyramidal neurons is tightly regulated by the density of low voltage-activated, non-inactivating K^+ current (Kv7/M-current) (Brown and Adams 1980; Halliwell and Adams 1982; Yue and Yaari 2004, 2006). Kv7/M-current (I_M) flows through M-type channels (KCNQ/M-channels) composed of homo- or heteromeric tetramers of five Kv7 (Kv7.1-7.5) subunits encoded by the KCNQ gene family. Kv7.2 and Kv7.3 are the major components of the M-type channel (Shah et al. 2002). I_M is inhibited through muscarinic receptor activation by acetylcholine and cholinomimetic drugs such as pilocarpine. We thus tested the hypothesis that the observed increase in neuronal excitability in CA3 pyramidal neurons, and the enhanced sensitivity to pilocarpine administration in GluK2^{-/-} mice, may all be explained by the modulation of inhibition of I_M by GluK2-containing KARs.

For this, we first performed whole cell voltage clamp recordings from CA3 pyramidal cells of GluK2^{+/+} FVB mice in the presence of TTX (1 μ M), ZD 7288 (20 μ M) and CdCl₂ (500 μ M) to pharmacologically suppress I_M -unrelated voltage-dependent conductances. The addition of Xe991 (50 μ M), a potent Kv7.2/7.3 channel blocker, allowed to reveal the I_M . Prior to, and twenty minutes after the administration of Xe991 in the bath, we applied a series of 10 voltage steps (5 mV increment) from the holding potential of -20 mV (Brown and Adams 1980) (Fig. 5A -top traces). The voltage steps elicited slow current relaxations representing the voltage dependent deactivation of I_M . These slow I_M currents were reduced by addition of 50 μ M of Xe991 (Fig. 5A bottom traces and graphs; from 116 $\pm$ 21 pA at -25 mV in control conditions (n=10) to 61.5 $\pm$ 6.5 pA at -25 mV, $p < 0.001$, 20 minutes after Xe991 application (n=10)), indicating successful pharmacological isolation of I_M . We then investigated the amplitude of I_M currents measured as the current subtraction between control and in presence of 10 μ M pilocarpine (20 minutes after the drug addition) in GluK2^{+/+} versus GluK2^{-/-} cells. Voltage dependent deactivation of I_M was not different in GluK2^{+/+} (n=11) and GluK2^{-/-} (n=10) CA3 pyramidal cells in control conditions (mean current amplitude 113 $\pm$ 18 pA at -25 mV for GluK2^{+/+} and 98 $\pm$ 10 pA at -25 mV for GluK2^{-/-}) (Fig. 5B -top graph and Fig. 5C -top graph and traces). However we observed that the I_M currents were significantly inhibited by pilocarpine at a concentration of 10 μ M in GluK2^{-/-} (n=10) neurons (from 98 $\pm$ 10 pA to 61 $\pm$ 13 pA at -25 mV), however the same concentration of pilocarpine has not been potent enough to significantly affect GluK2^{+/+} (n=9) neurons (from 113 $\pm$ 18 pA to 101 $\pm$ 21 pA -25 mV) (Fig. 5B -bottom graph and Fig. 5C -bottom graph and traces). Overall this experiment indicates that the cholinergic inhibition of I_M in CA3 pyramidal neurons is negatively modulated by GluK2-containing KARs.

Altered spike after potentials in GluK2^{-/-} cells after pilocarpine administration

Slowly activating outward K^+ currents like I_M act as a 'brake' on repetitive action potential discharges and, as such, regulate the excitability of various central and peripheral neurons

(Delmas and Brown 2005). In hippocampal principal cells I_M currents contribute to the spike after potentials i.e. after-hyperpolarizations (AHPs) and after-depolarizations (ADPs) (Yue and Yaari 2004; Caspi *et al.*, 2009; Mateos-Aparicio *et al.*, 2014). We thus tested the impact of GluK2 deletion on spike after potentials measured at +10 ms (ADP) and +100 ms (medium AHP, mAHP) after the spike in the presence of pilocarpine (10 μ M). The amplitude of the ADP and mAHP depends on the membrane potential at which the APs are generated (initial V_m : iV_m) (see Figure 6A). Here we based our analysis on spontaneous APs recorded in control and CA3-cKO pyramidal cells in the presence of pilocarpine. We first isolated spontaneous spikes regardless of the iV_m and measured ΔV_m (change in V_m) at +10 ms and +100 ms after the spike and averaged the value for each cell. We observed that in control conditions ΔV_m was higher in CA3-cKO cells at +10 ms (representing a larger ADP) (WT: 2.6 ± 3.3 mV ; cKO 5.9 ± 2.6 mV; $p=0.04$) (Figure 6B-C). Moreover, the ΔV_m was lower than zero at +100 ms (mAHP) only in WT condition but not in cKO. (WT: -1.4 ± 1.0 , $p=0.002$; cKO -0.5 ± 1.3) (Figure 6B, D). We then measured ΔV_m in reference to initial V_m , and we binned the values of ΔV_m from all cells within a genotype according to the initial V_m (2 mV bins) (Figure 6D). This provided a complex relationship showing that in control CA3 pyramidal cells, the ADP was revealed when the iV_m ranged between -60 and -55 mV (Figure 6E), and the mAHP was mainly observed with depolarized V_m (initial V_m above -45 mV) (Figure F). The relationship between ΔV_m and initial V_m was markedly different in CA3-cKO pyramidal cells (Figure 6E, F). First, the ADP was present and consistently larger than in control CA3 pyramidal cells for initial V_m above -55 mV. Second, a consistent mAHP was not observed in CA3-cKO pyramidal cells whatever the initial V_m (Figure 6F). The combination of enhanced ADP and reduced mAHP can explain the increased excitability of CA3 pyramidal cells with selective deletion of GluK2 in the presence of pilocarpine.

In order to complement these functional observations, we tested whether Kv7.2, a constituent of KCNQ/M-channels, was co-expressed with GluK2 in CA3 pyramidal cells. We analyzed the immunohistochemical distribution of GluK2 and Kv7.2 using antibodies (GluK2/K3 and Kv7.2 respectively) which have been previously tested for their specificity (Wyeth *et al.* 2014; Jenkins *et al.* 2015; Straub *et al.* 2016; Pablo and Pitt 2017). The experiment was conducted on brain sections from Thy1-GFP mice (Thy1-GFP-M) (Feng *et al.* 2000) which express GFP in sparse subsets of neurons within specific populations and have proven useful for labeling particular projections including mossy fibers. As expected, GluK2/K3 immunoreactivity was observed almost exclusively in the *stratum lucidum* (Fig. 7A, top panels). The GluK2/K3-related fluorescence intensity observed in brain sections ($n=6$) was on average 2.4 ± 0.3 folds higher in the *stratum lucidum* than in the *stratum radiatum* (Fig. 7A, top panels and graph). Kv7.2 immunoreactivity was high in the pyramidal cell layer of analyzed sections ($n=6$) but was also

clearly observed in *stratum lucidum* (Fig. 7A, bottom panels and graph). The pre- and postsynaptic compartments of mossy fiber synapses can be resolved using GFP fluorescence as a volume marker (Feng et al. 2000). Subsequent confocal microscopy revealed immunoreactivity for Kv7.2 in both giant mossy fiber boutons and in the postsynaptic thorny excrescence in CA3 pyramidal (Fig. 7B), indicating that GluK2-containing KARs and M-channels colocalize at mossy fiber synapses.

DISCUSSION

The acute administration of the neurotoxin kainate has long been known to trigger behavioural and electrophysiological seizures (Ben-Ari and Cossart 2000). Kainate administration in rodents has helped understanding the physiology of seizures and has been fruitful for the discovery of antiepileptic drugs (Rogawski 2006). However the fact that kainate is a high affinity ligand for KARs (Bettler and Mulle 1995) does not necessarily imply that KARs are involved in the complex etiology of TLE (Crépel and Mulle 2015). This work was initially designed to address the role of the different KAR subunits in the action of different convulsant drugs with distinct mechanisms of action. We demonstrate that none of the KAR subunits tested promote acute epileptic discharges induced by pilocarpine and by PTZ, two convulsants used to model acute epileptogenic activity (Turski et al. 1984; Löscher et al. 1991). Unexpectedly, we found that deletion of the GluK2 subunit increases the sensitivity to pilocarpine-induced seizures. Our results indicate that GluK2-containing KARs control the excitation of CA3 pyramidal cells induced by the muscarinic agonist pilocarpine by controlling the inactivation of KCNQ/M-currents and its role in shaping the spike afterpolarization (ADP and mAHP). The demonstration that GluK2/K3 and Kv7.2 immunoreactivity co-compartmentalizes at mossy fiber synapses further suggests a functional association between GluK2-containing KARs and pathways modulating KCNQ/M currents.

Our results first confirm that GluK2-containing KARs represent the main target for acute epileptic seizures induced by kainate (Mulle et al. 1998a). Because mouse strains differ from one another in their susceptibility to the actions of convulsants (Ferraro et al. 1995) and to seizure-induced excitotoxic cell death (Schauwecker 2012), we have tested pilocarpine and other chemoconvulsants in two different mouse genetic backgrounds for GluK2 deletion, in the C57Bl6 and FVB backgrounds. Our results indicate that GluK2 is the main target of the chemoconvulsant kainate independently of the genetic backgrounds. Our experiments also exclude GluK1 and GluK3 as epileptogenic targets of kainate (Fritsch et al. 2014). Likewise, it has been reported that the proconvulsant activity of systemic ATPA (a selective GluK1 agonist), but not of AMPA or kainate, is mediated by GluK1-containing KARs (Fritsch et al. 2014). The epileptogenic action of kainate can likely be attributed to a direct depolarization of principal neurons in the hippocampus and neocortex by activation of GluK2-containing KARs

(Mulle et al. 1998b; Bureau et al. 1999; Beed et al. 2009). Moreover, our data contribute to the demonstration that kainate likely targets GluK2/GluK5 KARs, the main KAR subtype in the brain (Wenthold et al. 1994). The GluK5 subunit conveys high affinity to kainate in heteromeric KARs (Bettler and Mulle 1995; Contractor et al. 2003), and is likely an obligatory component of GluK2-containing KARs in the hippocampus.

We found that genetically removing GluK1, GluK2, GluK3 or GluK5 neither prevented acute seizures triggered by the systemic administration of pilocarpine, which predominantly targets M1 muscarinic acetylcholine receptors, nor PTZ, which targets GABA_A receptors. Similarly, deletion of GluK1 or GluK2 does not elevate the threshold for acute seizures in the olfactory bulb kindling model (Fritsch et al. 2014). It should be stressed however, that GluK2/GluK5 KARs play a major role in recurrent epileptic seizures in the pilocarpine model of chronic TLE (Peret et al. 2014; Matsuda et al. 2016), albeit these receptors are not necessarily involved in the initial epileptogenic events. The involvement of aberrant synaptic GluK2/GluK5 KARs at recurrent mossy fiber synapses onto dentate gyrus cells has been clearly identified as a central mechanism for chronic epileptic discharges (Epsztein et al. 2005, 2010; Peret et al. 2014). Interestingly, even though GluK2^{-/-} mice display a markedly higher susceptibility to acute seizures induced by systemic pilocarpine administration, GluK2^{-/-} mice are protected against chronic seizures in the pilocarpine mouse model of TLE (Peret et al. 2014). This clearly indicates a disconnection between the facilitating role of KARs in the acute convulsant activity of pilocarpine, and the involvement of GluK2/GluK5 KARs in chronic epilepsy which relies on long-term reorganization of mossy fibers and which promote recurrent seizures reminiscent of TLE (Epsztein et al. 2005, 2010; Peret et al. 2014). Along these lines, it is worth mentioning that the genetic deletion of GluK2 does not prevent mossy fiber sprouting in the pilocarpine mouse model of chronic TLE. Overall, it is important to clarify that our data do not indicate any involvement of the cholinergic modulation of hippocampal neurons by GluK2 in TLE.

The higher susceptibility to acute seizures induced by systemic pilocarpine administration in GluK2^{-/-} mice was documented by the lower doses necessary to induce acute seizures and by the shorter latency to behavioural manifestations of seizures. Considering the relevance for CA3 in triggering epileptic discharges (Reyes-Garcia et al. 2018) and the major role played by GluK2-containing KARs in CA3 pyramidal cells (Carta et al. 2014), we directly analyzed the impact of pilocarpine administration on the activity of individual CA3 pyramidal cells in brain slices. Application of pilocarpine (10 μ M) in rat hippocampal slices triggers brief (200 ms) interictal-like activity, as well as more prolonged synchronous neuronal activity comparable to acute seizure-like discharges characterized by depolarizing CA3 pyramidal cells (Nagao et al. 1996; Rutecki and Yang 1998). Both synaptic and non-synaptic events appear to account for the generation of acute seizure-like discharges by pilocarpine (Rutecki and Yang 1998). In CA3 pyramidal cells recorded in a slice preparation, muscarinic receptor activation supports

persistent firing (Jochems and Yoshida 2013) and modulates glutamatergic transmission either directly or indirectly through depolarization and spike discharges in CA3 interneurons and dentate gyrus cells (Vogt and Regehr 2001). Consistent with studies describing the phasic and tonic activation of muscarinic receptors by acetylcholine in CA3, (Dasari and Gullledge 2011) pilocarpine evoked a gradual depolarization of CA3 pyramidal cells associated with an increased frequency of action potentials. These effects were markedly potentiated in slices from GluK2^{-/-} mice as well as in CA3 pyramidal with cell specific deletion of GluK2. Acetylcholine-mediated regulation of CA3 excitability is mainly mediated by M1 muscarinic receptors (Dasari and Gullledge 2011), a primary target for pilocarpine. Hence muscarinic regulation of excitability in CA3 is under the control of GluK2-containing KARs. We further extended the analysis using conditional KO mice (Marshall *et al.*, 2018) to show that selective removal of GluK2 from CA3 pyramidal cells is sufficient to trigger hypersensitivity to pilocarpine. This demonstrates that the interaction between M1 muscarinic receptors and KARs is a cell autonomous effect taking place in CA3 pyramidal cells and does not rely on indirect network interactions.

To get further insight into the mechanisms of this crosstalk between KARs and muscarinic receptor pathways, we studied Kv7/M-currents, non-inactivating K⁺ currents which are the main player in mediating the effects of M1 muscarinic receptor activation in neurons (Brown and Adams 1980; Hamilton *et al.* 1997; Shapiro *et al.* 2000), and in controlling neuronal activity in the hippocampus. Suppression of Kv7/M-currents has deleterious effects on neuronal excitability by initiating a transition from physiological network activity to temporal lobe seizure (Devaux *et al.* 2004; Qiu *et al.* 2007). Here we report that KARs modulate Kv7/M-currents inhibition in CA3 pyramidal neurons. Spike after polarization play (ADPs and AHPs) play an important role in the overall neuronal excitability and these are modulated by M1 muscarinic receptors through activation of KV7/M currents (Yue and Yaari 2004; Gu *et al.* 2005) in CA1 pyramidal cells. We found that in the presence of pilocarpine the ADP is increased in CA3-cKO pyramidal cells, at all initial V_m from -35 to -55 mV. Conversely, whereas the mAHP was observed for an initial V_m above -45 mV in control CA3 pyramidal cells, the mAHP was not consistently measured in CA3-cKO pyramidal cells. The conjunction of an increased ADP and a decreased mAHP, together with an increased input resistance due to Kv7 closing (in agreement with Sun and Kapur 2012) likely participates in the hyperexcitability of CA3 pyramidal cells in the absence of GluK2. Overall GluK2-containing KARs participate in the modulation of neuronal excitability induced by cholinergic agonists in CA3 pyramidal cells. The genetic deletion of GluK2 leading to loss of control on Kv7/M-currents seems to potentiate the generation of seizures by pilocarpine within hippocampal circuits.

The molecular mechanisms explaining the interaction between GluK2 and muscarinic pathways regulating Kv7/M-currents, and potentially between KARs and muscarinic AChRs,

need to be elucidated. The mechanism of Kv7/M-currents inhibition by activation of M1 AChRs is indirect and requires G-protein activation through second messengers (Delmas and Brown 2005). KARs are known to exert a variety of metabotropic actions, independent of their ionotropic function (Lerma and Marques 2013). Among these actions, KARs likely comprised or GluK2/GluK5 subunits increase neuronal excitability of CA3 pyramidal cells (Fisahn 2005; Ruiz et al. 2005), by reversible inhibition of the slow Ca^{2+} -activated K^+ current (slow AHP) through a G-protein-coupled mechanism. Consequently removing GluK2 (hence GluK2/GluK5) decreases KAR mediated increase in neuronal excitability (Ruiz et al. 2005; Sachidhanandam et al. 2009). Interestingly, a recent study has shown that direct application of kainate or trains of synaptic stimulation reduce the postburst AHP in pyramidal neurons of the piriform cortex through activation of GluK2-containing KARs, in control animals but not in animals which have learned a complex olfactory task (Chandra et al. 2019).

Activation of mAChRs by pilocarpine in slices significantly enhances KAR currents and excitotoxicity mediated by GluK2/GluK5 (but not homomeric GluK2), and potentiates mossy fiber axon excitability (Benveniste et al. 2010). In contrast, we observe that pilocarpine-induced increase in neuronal excitability is enhanced in the absence of GluK2, indicating that the two mechanisms are not linked. Interestingly, P2X7R knockout mice similarly show enhanced susceptibility to seizures induced by pilocarpine, despite having similar levels of hippocampal M1 mAChR expression (Kim and Kang 2011). This hypersensitivity to pilocarpine does not involve GABAergic or glutamatergic transmission, but appears to depend on a process mediated by PKC via intracellular Ca^{2+} release. Overall our data suggest that GluK2-containing KARs may act as a negative regulator of the actions of mAChRs on the excitability of neural circuits. It would certainly be important to explore the physiological conditions under which KARs and mAChRs, activated by their endogenous ligands, interact to regulate the activity of hippocampal circuits *in vivo* in physiological conditions.

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FIGURE LEGENDS

Figure 1 - Behavioral responses to the systemic administration of proconvulsants in KAR mutant mice

A-C) Six to 16 mice representing different KAR mutants were intraperitoneally injected with kainate (A), pilocarpine (B), or PTZ (C). For kainate and pilocarpine administration the response varied from no effect (stage 0) and mild manifestation (stages 1,2,3) up to maximum effect (stage 4) and lethality (stage 5). For PTZ the response varied from no effect (stage 0) and mild manifestation (stages 1, 2, 3, 4, 5) up to maximum effect (stage 6) and lethality (stage 7). Note that no behavioral effect of kainate injection was observed in GluK2 and GluK5 knock-out mice, independently of the genetic background for GluK2^{-/-} mice. On the other hand GluK2^{-/-} mice were more prone to acute behavioural seizures in response to pilocarpine administration as compared to controls and all other mutant mice. No difference was observed within KAR mutant mice in response to PTZ injection.

Figure 2 - Seizure latency, excitotoxicity, and survivability in GluK2^{+/+} and GluK2^{-/-} mice in response to systemic pilocarpine administration

A) Two control and two experimental groups (9 to 10 mice each) were injected with pilocarpine according to the scheme. B1-B2) Graphs representing the latency to the appearance of the first tonic-clonic convulsion (stage 3 seizure) and to the beginning of the *status epilepticus* (stage 4 seizure), as well as percentage of survivors during the experiment in both control and GluK2^{-/-} mice. Note that application of pilocarpine (265 mg/kg) induced stage 3 and 4 seizures much faster and lead to higher mortality in GluK2^{-/-} mice as compared to control mice (B1, left graph). When the dose of pilocarpine was reduced (200 mg/kg), only moderate effects (no stage 3 or 4 seizure) were observed in control mice in contrast to marked effects observed in GluK2^{-/-} mice (B1, right graph). C) Fluoro-Jade positive neurons in the hippocampus of GluK2^{+/+} and GluK2^{-/-} mice 24h after intraperitoneal pilocarpine injection. The density of Fluoro-Jade positive cells in different regions of hippocampus was calculated for both control and experimental group (graph). Note the prominent excitotoxicity caused by pilocarpine (200 mg/kg) in GluK2^{-/-} mice in contrast to control mice.

Figure 3 - Pilocarpine action in CA3 pyramidal cells in slices from GluK2^{+/+} and GluK2^{-/-} mice

A) Perfusion of pilocarpine into the slice depolarized CA3 pyramidal cells. A concentration of pilocarpine (10 μ M) which moderately affected GluK2^{+/+} neurons (dark blue trace and graph) lead to spike discharges in GluK2^{-/-} neurons (light blue trace and graph). B) Synaptic transmission in WT and GluK2-cKO CA3 neurons (slices from GluK2^{fl} mice infected with viral vector harboring GFP or Cre-GFP). *Left*, stimulation of mossy fibers triggers EPSCs with

amplitude increasing with frequency (1 Hz vs 0.1Hz). *Right*, KAR-mediated EPSCs are isolated at 3 Hz (red traces). C) KAR mediated currents were not measurable in GluK2-cKO CA3 neurons. D) Hyperpolarizing and depolarizing current steps under control conditions and 20 minutes after 10 μ M pilocarpine addition to the bath. Pilocarpine increases spike discharge in both genotypes but the effect is greater in GluK2-cKO CA3 neurons (red traces and bottom graphs). E) The firing rate increases after pilocarpine application. This increase is larger in GluK2-cKO CA3 neurons. F) The hyperpolarizations are greater after application of pilocarpine and even larger in GluK2-cKO CA3 neurons. G) The input resistance increases after pilocarpine application. This increase is larger in GluK2-cKO CA3 neurons.

Figure 4 - Frequency of evoked action potential firing does not differ between GluK2^{+/+} and GluK2^{-/-} CA3 neurons under influence of increased extracellular K⁺ levels

Application of depolarizing current steps at the beginning (control conditions) and 20 minutes after ACSF exchange did not reveal any significant difference in the frequency of action potential firing between both genotypes (traces and top graphs) and in the input resistance (bottom graphs).

Figure 5 - Involvement of GluK2-containing KARs in the modulation of M-currents

A) Voltage/current relation demonstrates the sensitivity of the recorded current to Xe991 in GluK2^{+/+} cells (top traces and top graph). Xe991-mediated reduction of the current amplitude in GluK2^{+/+} cells 20 minutes after the drug application (bottom traces and bottom graph) indicates that I_M is a significant component of the analyzed response. B) Voltage-dependent deactivation of the I_M did not differ significantly between GluK2^{+/+} and GluK2^{-/-} neurons in control conditions (top graph). However, 10 μ M pilocarpine significantly inhibited I_M in GluK2^{-/-} neurons but not in GluK2^{+/+} neurons (bottom graph) 20 minutes after the drug application. C) Pilocarpine-mediated effect on the I_M current amplitude 20 minutes after the drug application to GluK2^{+/+} (top traces and graph) and GluK2^{-/-} cells (bottom traces and graph).

Figure 6 - Altered spike after potentials in GluK2^{-/-} cells after pilocarpine administration

A) Representative spontaneous AP recorded from CA3 pyramidal cell at -60 (left) or -50 (right) mV. The dotted lines extend the membrane potential prior AP (initial V_m: iV_m). For a RMP of -60 mV, an after depolarization (ADP, a Δ V_m positive to iV_m) is observed in control condition +10 ms after the peak of the AP. For a RMP of -50 mV, an after hyperpolarization (AHP, a Δ V_m negative to iV_m) is observed in control condition after the peak of the AP. A fast and a moderate component of AHP (mAHP, at +100 ms after the AP peak) are distinguished. B) Representative spontaneous AP recorded from CA3 pyramidal cell under pilocarpine condition at -50 mV in WT (left) or in GluK2-cKO neurons (right). An ADP is observable in GluK2-cKO CA3 but not in WT pyramidal cells at +10 ms. A small mAHP is observed at +100 ms after the

AP peak. C) Bar plot combined of scatter values from individual neurons of the ΔV_m at +10 ms after the AP peak. The ΔV_m were calculated from all spontaneous APs, whatever iV_m , and averaged per cell (upon pilocarpine treatment). In average a positive ΔV_m is observed in both WT and GluK2-cKO CA3 but the amplitude is larger in GluK2-cKO CA3 neurons. D) Bar plot combined of scatter values from individual neurons of the ΔV_m at +100 ms after the AP peak. The ΔV_m were calculated from all spontaneous APs, whatever iV_m , and averaged per cell (upon pilocarpine treatment). In average a negative ΔV_m (different than 0) is observed in WT but not in GluK2-cKO CA3 neurons. E, F) ΔV_m plotted in function of iV_m at +10 (E) or +100 (F) ms after the AP peak. Whereas a positive ΔV_m (ADP) is observed below -55mV in all conditions, this ADP is observed above -55 mV only in GluK2-cKO CA3 neurons (E). A negative ΔV_m (AHP) is observed above -45mV in WT but not GluK2-cKO CA3 neurons (F).

Figure 7 - Co-expression of GluK2 and Kv7.2 in *stratum lucidum*

A) GluK2/K3 (top panels) and Kv7.2 (bottom panels) immunoreactivities in conjunction with EGFP expression under Thy1 promoter showing the pattern of expression of these proteins in the hippocampus. The presence of GluK2/K3 and Kv7.2 subunit in the *stratum lucidum* indicates association of both channels with mossy fiber pathway. The expression profile of both proteins in the hippocampus is presented in the graph as the average fluorescence intensity within distinct regions of CA3 (*stratum radiatum*, *stratum lucidum*, and *stratum pyramidale*) normalized to *stratum radiatum*-derived average fluorescence (graph). B) Single confocal plane images and 3D reconstructions of image stacks demonstrate colocalization of M-channel with giant mossy fiber boutons (left panels) and thorny excrescence (right panels). Localization of Kv7.2 in both pre- and postsynaptic compartments suggests co-expression of KARs and M-channel at mossy fiber synapses.

A

B

C

Figure 1

Figure 2

Figure 3

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Figure 5

Figure 6

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