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ORIGINAL ARTICLE

Repeated Application of 4-Aminopyridine Provoke an Increase in Entorhinal Cortex Excitability and Rearrange AMPA and Kainate Receptors

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Abstract Entorhinal cortex is a highly epilepsy-prone brain region. Effects of repetitive seizures on ionotropic glutamate receptors (iGluRs) were investigated in rat entorhinal cortex slices. Seizures were induced by daily administration of 4-aminopyridine (4-AP). Electrophysiological, pharmacological and histological investigations were carried out to determine changes in synaptic efficacy and in sensitivity of iGluRs due to recurring seizures. Repeated 4-AP-induced seizures increased the amplitude of evoked synaptic field responses in rat entorhinal cortical slices. While vulnerability to inhibition of AMPA receptors by the specific antagonist GYKI 52466 was slightly reduced, responsiveness to NMDA receptor antagonist APV remained unaffected. Testing of bivalent cation permeability of iGluRs revealed reduced Ca^{2+}-influx through non-NMDA receptors. According to the semi-quantitative histoblot analysis GluA1–4, GluA1, GluA2, GluK5, GluN1 and GluN2A subunit protein expression differently altered. While there was a marked decrease in the level of GluA1–4, GluA2 and GluK5 receptor subunits, GluA1 and GluN2A protein levels moderately increased. The results indicate that brief convulsions, repeated daily for 10 days can increase overall entorhinal cortex excitability despite a reduction in AMPA/kainate receptor activity, probably through the alteration of local network susceptibility.

Keywords Seizure · Brain slice · 4-Aminopyridine · Entorhinal cortex · Glutamate receptor

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Introduction

The imbalance of excitatory (glutamatergic) and inhibitory (γ-amino-butyric acid (GABA)-ergic) neurotransmission in the forebrain is a key feature of epilepsy. It is widely accepted that ionotropic glutamate receptors (iGluRs) are involved in the development of various forms of epilepsy. The iGluRs are glutamate-gated cation channels composed of four subunits (Traynelis et al. 2010). Based on their sequence homology, electrophysiological properties and pharmacological selectivity, iGluRs are subdivided into three main subtypes: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), kainate (KA) and *N*-methyl-D-aspartate (NMDA) type receptors (Traynelis et al. 2010). AMPA receptors (AMPARs) mediate fast synaptic transmission in the central nervous system (CNS) and they are key components of the modifiable synaptic response. AMPARs are composed of the GluA1–4 subunits, which can form functional heteromeric receptors. Most native AMPARs contain both the GluA2 subunit and either GluA1, GluA3 or GluA4 (Traynelis et al. 2010). Kainate receptors (KARs) are key players in the modulation of neuronal network activity throughout the CNS (Lerma 2003). While other AMPARs and NMDA receptors (NMDARs) operate mainly at postsynaptic sites, KARs are both presynaptic and postsynaptic, where they modulate

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neurotransmitter release and effectiveness of excitatory neurotransmission (Jane et al. 2009; Lerma 2003). Molecular cloning has identified five KAR subunits, named GluK1–5 (Traynelis et al. 2010). Functional receptors are usually formed from heteromeric combinations of subunits and the most common postsynaptic KAR subunit combination is GluK2/GluK5 (Lerma 2003). NMDARs contribute to the late component of synaptic response and function as coincidence detectors (Collingridge et al. 2004; Traynelis et al. 2010). NMDARs are responsible for a wide range of postsynaptic functions, including changes in synaptic effectiveness when long-term potentiation (LTP) or long-term depression (LTD) develops (Collingridge et al. 2004). Excessive activation of NMDARs leads to pathological processes (e.g. excitotoxic injury) in a number of acute and chronic neurological disorders (Lau and Tymianski 2010). NMDARs are obligate heterotetramers formed by the assembly of GluN1 subunits with GluN2A–D and GluN3A/B (Traynelis et al. 2010). Pathological glutamate receptor function may underlie many neurological disorders (Lau and Tymianski 2010).

Temporal lobe epilepsy (TLE) is one of the most common forms of intractable focal epilepsies (Chang and Lowenstein 2003). Ex vivo brain slice experiments support the idea that seizures are likely to be generated in the entorhinal cortex and subsequently spread to the hippocampus in TLE (Armand et al. 1999). While several animal models were introduced for the study of TLE, most widely kainate or pilocarpine are applied to induce epileptiform seizures. These convulsants provoke status epilepticus (SE) soon after the treatment (Cavalheiro et al. 1991; Turski et al. 1983). Kainate provokes SE and after a silent period of 2–3 weeks, spontaneous motor limbic seizures are generated (Nadler 2003). In the pilocarpine model, spontaneous seizure episodes appear following a 4–44 days-long silent period as the consequence of neurodegenerative processes (Scorza et al. 2009). Neuronal loss and rearrangement of synaptic connections are characteristic features of these models (Choi et al. 2007; Fisher 1989; Nadler et al. 1978).

A moderate dose of intraperitoneally applied 4-AP, a potassium channel blocker, also induces acute seizures in rats. In contrast with kainate and pilocarpine treatment, low doses of 4-AP does not induce chronic spontaneous seizures or cell damage (Vizi et al. 2004). The apparent lack of neuronal loss following repeated 4-AP convulsions renders this model suitable for the study of genuine in vivo molecular alterations developing after repeated seizures. On the other hand, 4-AP administered directly to the hippocampus causes acute seizures and severe cell loss, especially in the CA1 and CA3 hippocampal regions (Pena and Tapia 2000). In this case, however, the local concentration of 4-AP is relatively high. 4-AP applied directly to brain slices ex vivo also induces seizure-like events that can be inhibited by both NMDAR and AMPAR antagonists (Doczi et al. 1999; Gulyás-Kovács et al. 2002). Both ex vivo and in vivo 4-AP treatment causes an elevation in the transcription of certain immediate-early genes (such as c-fos and c-jun) suggesting that seizures may cause changes in neuronal gene expression in the affected brain areas (Borbely et al. 2006; Mihaly et al. 2005).

Previous ex vivo studies revealed brain region-specific changes in glutamatergic neurotransmission in the rat neocortex and hippocampus following repeated 4-AP-induced seizures (Borbely et al. 2009; Vilagi et al. 2009). In the present study, the effects of repeated 4-AP-induced seizures on the excitability of the entorhinal cortex and changes in iGluRs are investigated. Evoked field potentials (EFPs) were tested to evaluate changes in the basic excitability together with the pharmacological sensitivity to specific AMPAR and NMDAR antagonists (GYKI 52466 and APV, respectively). Kainate-induced Co^{2+}-uptake assay was performed to evaluate the changes induced by the repeated 4-AP treatment in the Ca^{2+}-permeability of AMPARs and KARs. Alterations in iGluR protein expression levels were analysed by semi-quantitative histoblots (Kopniczky et al. 2005; Tonnes et al. 1999) using selective antibodies to GluA1, GluA2, GluA1-4, GluK5, GluN1 and GluN2 iGluR subunits. Our results indicate that repeated brief convulsions lead to an increase of entorhinal cortex excitability despite an overall reduction in AMPAR/KAR activity.

Materials and Methods

Experiments were performed on adult, male Wistar rats (150–280 g, Charles Rivers, Budapest, Hungary). All procedures were approved by the regional Animal Ethics Committees in Budapest (p.n.: 22.1/829/003/2007) and Szeged (p.n.: I./01508/2011) and by the European Communities Council Directive of 1986 (86/609/EEC). Rats were kept under constant 12 h light/dark cycle and controlled temperature (22 ± 2 °C). Standard pellet food and tap water were available ad libitum. All chemicals were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary) or Merck Ltd. (Budapest, Hungary) unless it is stated otherwise. GYKI 52466 was the kind gift of Dr. I. Tarnawa (Richter Gedeon Plc, Budapest, Hungary).

Seizure Induction in Rats

4-AP, dissolved in physiological saline, was injected every day intraperitoneally for 12 consecutive days. The starting dose was 4.5 mg/kg. After the injection, the animals were monitored for 2–2.5 h until they fully recovered from

seizures. Seizures were characterised according to the widely used five-stage Racine scale (Racine 1972). If the rats did not show a stage 5 seizure after the third day of treatment, the dose was increased by 5 %, and it was further increased by 5 % on any day when a stage 5 seizure failed to develop on the previous day. The maximal dose was 6.65 mg/kg. This group of animals is referred to as *seizured group*. Control rats received physiological saline intraperitoneally (0.5 ml) for 12 days, and were referred to as *normal group*. The maximum injected volume of 4-AP or saline was always less than 1 ml.

Slice Preparation and Electrophysiological Recording

Electrophysiological recordings were carried out on 48 slices from 18 rats, 8 slices were used in each experimental group. Animals were decapitated in deep chloral-hydrate anaesthesia (350 mg/kg), brains were quickly removed and horizontal plane slices (400 µm thick) of the entorhinal cortex were cut with a vibratome. Brain slices were left for 1 h to stabilise in the incubation solution containing *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer: (in mM): 120 NaCl, 20 $NaHCO_3$, 2 KCl, 1.25 KH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 6.7 HEPES, 2.6 Na-HEPES, pH 7.2–7.3). This solution was saturated with carbogene (5 % CO_2, 95 % O_2) at room temperature (20–22 °C). Then slices were transferred to a Haas-type recording chamber (Experimetria Ltd., Budapest, Hungary) which was perfused with standard artificial cerebrospinal fluid (ACSF; 1.5 ml/min) using a peristaltic pump (Heidolph PD 5101, Schwabach, Germany). The composition of ACSF was (in mM) 126 NaCl; 26 $NaHCO_3$; 1.8 KCl; 1.25 KH_2PO_4; 1.3 $MgSO_4$; 2.4 $CaCl_2$; 10 glucose, pH 7.1–7.3. The solution was saturated with carbogene at 33 ± 1 °C before application.

Glass microelectrodes filled with 1 M NaCl (8–10 MΩ) were positioned as recording electrodes into layer III of the lateral entorhinal cortex, while bipolar tungsten stimulating electrodes were positioned below the recording electrodes at the border of the white and grey matters. Duration of the stimulation square voltage pulses was 100 µs and the amplitude was gradually varied between threshold and supramaximal values. Threshold (T) means the stimulation strength when the evoked response just appears, and T was introduced for better comparability of the recorded data. Signals were amplified 1,000-fold with a preamplifier and an Axoclamp 2A amplifier (Axon Instruments Inc., Union City, CA), A/D converted and recorded with the SPEL Advanced Intrasys computer program (Experimetria Ltd., Budapest, Hungary).

An evoked response was typically made up of an early synaptic (referred to as N1) component, the latency of which was always above 4.5 ms (Tolner et al. 2007). This wave was followed by a series of longer latency peaks with smaller amplitude referred to as late components (Fig. 1a). Sometimes, but not always, a smaller, very early non-synaptic peak (N0) also appeared, showing antidromic activation. In the present evaluation, the amplitude of the early (N1) synaptic component of EFP was systematically analysed, while the late component of EFP (N2) was not involved in detailed analysis due to its large variability.

The viability of each slice was tested before the beginning of recording procedure by applying single pulse stimulation and monitoring characteristic field responses. If the absolute amplitude of N1 was smaller than 1 mV at approximately 4T intensity, the slice was excluded from further experiments. Slices were continuously stimulated with 2T stimuli at a frequency of 0.1 Hz, to stabilise the response amplitude. At least 15 min after placing the slices into the recording chamber, the exact stimulus threshold (1T) was determined. The recording session was started only if all these tests were passed. At the beginning of recording (0 min), an input–output curve (I–O curve, stimulus intensity—amplitude of EFP) was measured from 1 to 4T stimulus intensity in 0.5T steps. During the subsequent incubation phase, continuous stimulation was applied with 0.1 Hz frequency and 2T intensity. After 10 or 30 min, another I–O curve was recorded. To analyse the pharmacological sensitivity of particular iGluRs, we applied either AMPAR antagonist (40 µM 1-(aminophenyl)-4-methyl-7,8 methylendioxy-5*H*-2,3-benzodiazepine (GYKI 52466)) for 30 min or NMDAR antagonist (25 µM D-2-amino-5-phosphonopentanoic acid (D-APV)) for 10 min. The antagonists were dissolved in ACSF, the applied concentration and the duration of treatments with the antagonists was established by our previous studies (Gulyás-Kovács et al. 2002; Vilagi et al. 1996). Recorded data were analysed using the SPEL Advanced Intrasys computer program (Experimetria, Budapest, Hungary). In each experiment, the amplitude of each EFPs was normalised to the EFP evoked in 0 min at 4T stimulus intensity for better comparability of the changes.

Co^{2+}-Permeability of Non-NMDARs

Brain slices from normal ($n = 9$) and seizured ($n = 10$) animals were used to test alterations in Co^{2+} uptake. Co^{2+} can pass through activated non-NMDARs that are also permeable to Ca^{2+}, but not through NMDARs (Mayer and Westbrook 1987).

Slice preparation was carried out in the same way as described for electrophysiological recordings. The thickness of the slices was 250 µm. From each rat, 8–10 entorhinal cortex slices were stained and evaluated. The procedure was performed as described previously (Pruss et al. 1991). Briefly, slices were incubated in a Ca^{2+} free

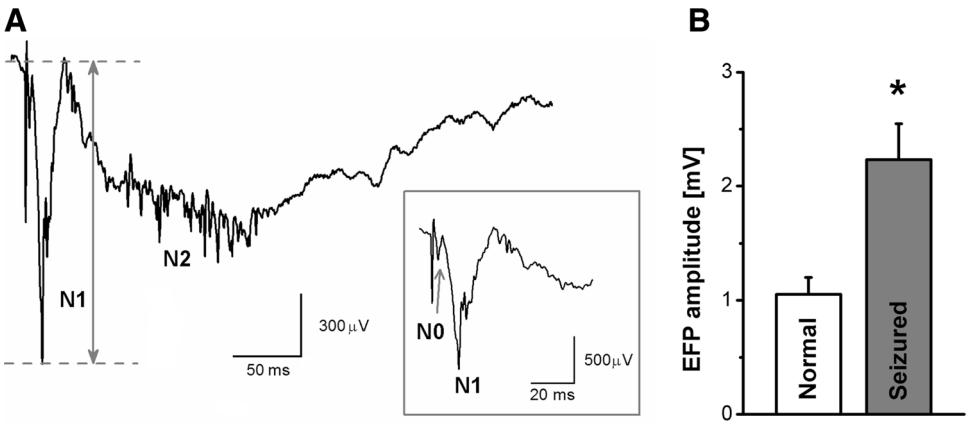

Fig. 1 Characteristic EFP in entorhinal cortex and changes in general neuronal excitability. **a** Characteristic trace of evoked response from entorhinal cortex. The non-synaptic N0 component was followed by the early N1 component from seizured rat, which is thought to be the first synaptic component of the evoked response, was determined as the negative peak measured with at least 4.5 ms delay. The later component (N2) of the evoked response was quite heterogeneous in appearance, and lasted for up to several hundreds of milliseconds. *Small inset* shows the N0 and N1 components. **b** The alteration of general neuronal excitability as a measure of absolute EFP amplitude in normal and seizured groups ($n = 5$ and $n = 6$ respectively). In seizured group, the excitability was significantly higher than in normal group. $*p < 0.05$

incubation solution for 5 min before placement into a Co^{2+} uptake buffer (13 mM sucrose, 57.5 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 12 mM glucose, 10 mM HEPES, pH 7.4) supplemented with 5 mM $CoCl_2$ and 100 µM kainate. Incubation lasted for 20 min at room temperature (20–22 °C). Control slices serving as staining background were incubated in the same solution without kainate. After the stimulated Co^{2+} uptake, slices were rinsed once in the uptake buffer and then incubated in the same buffer containing 2 mM ethylenediaminetetraacetic acid (EDTA) to remove non-specifically bound Co^{2+}. Slices were then rinsed twice in the uptake buffer, and Co^{2+} was precipitated through incubation in a 0.12 % $(NH_4)_2S$ solution for 5 min. During this procedure, dark CoS precipitates were formed in the cells. Slices were then fixed in 4 % paraformaldehyde for 30 min and mounted to glycerine-coated slides for further computer analysis. The Co^{2+}-stained slices were analysed with an Olympus CH-2 microscope equipped with an Olympus Camedia C4040-Zoom digital camera connected to a PC running AnalySIS 3.2 Docu software (Olympus Soft Imaging Solution GmbH, Münster, Germany). Pictures were digitalised at a fourfold magnification and converted to 8-bit grey scale images. Staining intensity was measured in arbitrary units. One data point corresponded to the mean of 50 recorded points (50× 300–600 pixels/point). Optical density (OD) of a 50 × 100 pixel area in the corpus callosum was determined in each slice as background, and data were always corrected with this OD value. The corrected OD values of sham-treated normal slices were subtracted from the corrected OD values of kainate-stimulated slices to reduce the bias from non-specific staining. OD values of slices from normal and seizured groups of animals were compared, relative change was given as a percentage of OD value originating from slices of normal group. For more accurate comparison of the corresponding cortical layers, the distance between the pial surface and the subcortical white matter was divided into 20 equal divisions which were correlated to cortical layers using corresponding Nissl-stained sections. The borders of the layers were as follows: I: division 1–5, II: 6–7 division, III: 8–12 division, IV: 13–14 division, V: 15–17 division, VI: 18–20 division.

Histoblot Analysis of AMPAR and KAR Subunit Proteins

Rats were killed following the final 4-AP treatment, and used to determine changes in the distribution of different AMPAR subunit proteins, using an in situ blotting technique (Gallyas et al. 2003; Kopniczky et al. 2005; Tonnes et al. 1999). Animals were deeply anesthetised with diethyl ether, decapitated and the brains were quickly frozen in isopentane and stored at −80 °C until sectioning. Horizontal plane cryostat sections (10 µm) were cut and mounted on glass slides. The sections on the glass were pressed against nitrocellulose membranes, which were previously moistened with 48 mM Tris-base, 39 mM glycine, 2 % (w/v) sodium dodecyl sulphate (SDS) and 20 % (w/v) methanol for 15 min at room temperature (20–22 °C). After blocking in 5 % (w/v) non-fat dry milk in phosphate-buffered saline (PBS), nitrocellulose membranes were DNase I-treated (5 U/ml), washed and incubated in 2 % (w/v) SDS, 100 mM β-mercaptoethanol in 100 mM Tris–HCl (pH 7.0), for 60 min at 45 °C to remove

adhering tissue residues. After excessive washing, blots were reacted with affinity-purified subunit specific antibodies (0.5 μg/ml) in blocking solution overnight at 4 °C. The following primary antibodies were used: rabbit anti-GluA1; rabbit anti-GluA2 (Chemicon; 1 μg/ml); rabbit anti-GluA1–4 (pan-AMPAR), (Pickard et al. 2000); rabbit anti-GluK5 (Upstate; 1:500). The bound primary antibodies were detected with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody (Tonnes et al. 1999). To facilitate the identification of structures and cell layers, adjacent cryostat sections were stained with cresyl violet. Digital images were acquired by scanning the membranes using a desktop scanner, and the data were captured as grey scale images for evaluation. Evaluation of images was carried out according to the method described at cobalt uptake determination using the same camera and image processing software.

Statistical Analysis

To compare I–O curve datasets obtained with control and treated slices, two-sample unpaired Student's t test was used. Homogeneity of variances was examined by the two-sample F test. The data are presented as mean ± SEM ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Results

Seizure Behaviour

Intraperitoneal (i.p.) administration of 4-AP (4.5–6.21 mg/kg) caused epileptiform seizures ranging from stage 3 to 5 on Racine's scale (Racine 1972). Stage 5 generalised seizures were induced on 5.0 ± 1.5 days ($n = 27$) out of 12 days, the development of seizures was random during the 12-day treatment. When this stage developed, the latency of the first seizure event was 26.6 ± 5.4 min ($n = 27$), and sometimes the first seizure was followed by a second generalised seizure within the first hour after the 4-AP injection. The mean delay between the two convulsions was 16.2 ± 4.9 min ($n = 27$). The symptoms of 4-AP seizures were described previously in detail (Mihaly et al. 1990; Szakacs et al. 2003; Weiczner et al. 2008), and analysed with electroencephalography (EEG) in our laboratories (Kopniczky et al. 2005; Mihaly et al. 2005).

Increased General Neuronal Excitability

General neuronal excitability was characterised with the amplitude of the EFP evoked by electrical stimulation, where changes in the first component (N1 peak) were measured in our experiments (Fig. 1a). The excitability was tested at 2T stimulus intensity after the stabilisation of EFP amplitude. Pronounced differences were detected in evoked responses: the normal group showed a 1.05 ± 0.15 mV field response, while the seizured group showed a 2.23 ± 0.31 mV response. ($*p = 0.011$, $n = 10$ and $n = 11$ for normal and seizured groups, respectively). This indicates a 110 % higher excitability in the seizured group compared to normal animals (Fig. 1b).

The Effect of the AMPAR Antagonist was Reduced in the Seizured Slices

The pharmacological sensitivity of the N1 component of the EFP was tested with the selective AMPAR antagonist, GYKI 52466 (Tarnawa et al. 1990). GYKI 52466 was applied in 40 μM final concentration for 30 min in the perfusion solution. I–O curve and EFP amplitude at 2T stimulation were determined in normal and seizured groups before and immediately after finishing GYKI 52466 application. Each EFP amplitude value was normalised for the EFP amplitude of maximal stimulation intensity (4T) recorded at 0 min. The slices of the normal group showed pronounced inhibition of the EFP in the presence of AMPAR antagonist GYKI 52466 (Fig. 2a). The inhibitory effect of GYKI 52466 was also significant in the seizured group, however, the observed decrease of relative EFP was smaller than in slices of the normal group, despite the fact that the control values of relative EFP were similar in both animal groups (Fig. 2a). GYKI 52466 was able to suppress the EFP at every stimulus intensity in either normal or seizured groups.

The inhibitory effect of GYKI 52466 was further analysed in normal and seizured groups, at 2T stimulus intensity. In the normal group, the amplitude of the N1 component of the EFP was reduced in the presence of GYKI 52466 (Fig. 2b). At the end of the 30 min incubation period the relative amplitude of EFPs at 2T intensity was 100.0 ± 10.6 % in control slices (Fig. 2c; $n = 5$) which was significantly reduced to 35.6 ± 9.1 % relative amplitude in the presence of GYKI 52466 (Fig. 2c; $n = 5$, $**p = 0.002$, compared to control). In the seizured group, GYKI 52466 also reduced the amplitude of the N1 component of the EFP (Fig. 2d). The relative amplitude of EFPs at 2T stimulus intensity was 115.0 ± 7.8 % (Fig. 2e; $n = 6$) in control slices at 30 min, and reduced to 74.1 ± 8.9 % (Fig. 2e; $n = 5$, $**p = 0.007$, compared to control) in GYKI 52466-treated slices.

The relative amplitude of EFPs showed no difference in the control slices of the normal and the seizured group ($p = 0.275$), however, there was a significant difference between the GYKI 52466-treated slices of the normal and the seizured animals ($*p = 0.017$). These results indicate that the inhibition of AMPARs by GYKI 52466 was significantly reduced in the seizured group.

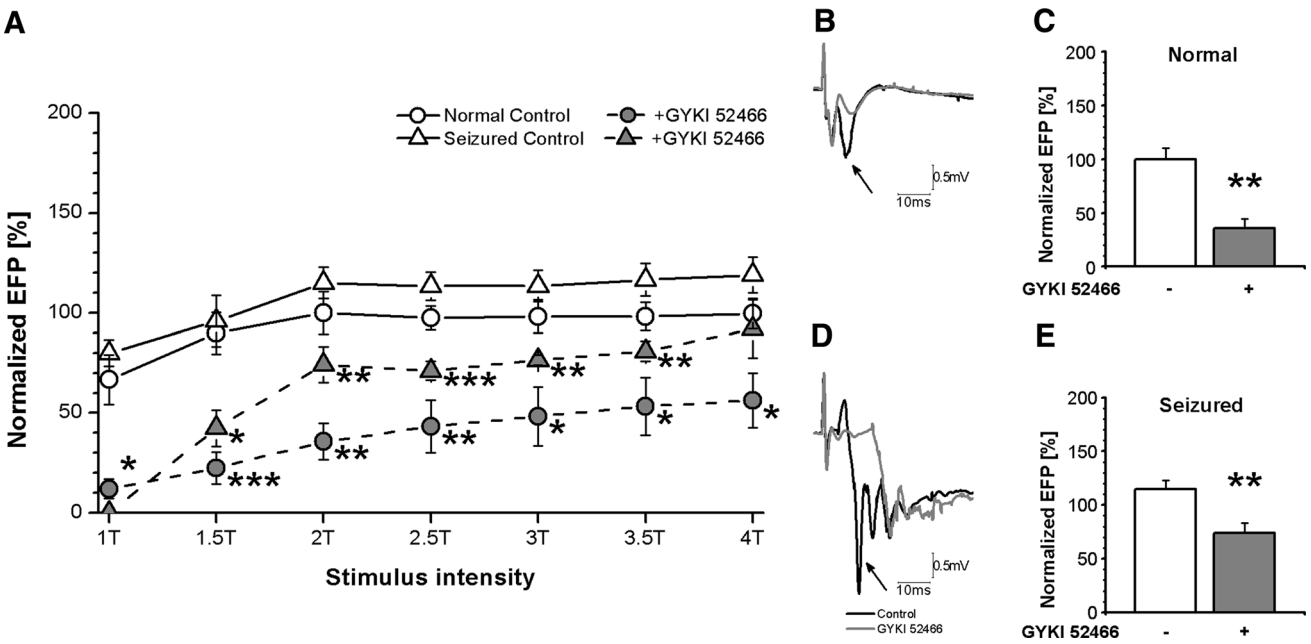

Fig. 2 GYKI 52466 inhibitory efficacy in normal and seizured rats. **a** Relative EFP value of N1 component was plotted against stimulus intensity showing the effect of the AMPAR antagonist GYKI 52466 (40 μM) in slices from normal and seizured rats. GYKI 52466 effectively inhibited in evoked responses normal group at every intensity and it was also effective in brain slices obtained from seizured rats. However, the relative inhibition was less prominent in the seizured group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **b** Characteristic EFP traces of GYKI 52466 effect in normal group. The *arrow* indicates the N1 component of EFP. **c** Antagonistic effect of GYKI 52466 on EFP, induced by 2T stimulus intensity in slices of normal group. The relative EFP amplitude following GYKI 52466 treatment was significantly reduced ($n = 5$, **$p = 0.002$). **d** Characteristic EFP traces of GYKI 52466 effect in seizured group. The ↑ indicates the N1 component of EFP. **e** Antagonistic effect of GYKI 52466 on EFP, induced by 2T stimulus intensity in slices of seizured group. The application of GYKI 52466 significantly reduced the relative N1 amplitude ($n = 5$, **$p = 0.007$)

The Effect of the NMDAR Antagonist was Mildly Increased After 4-AP Treatment

The inhibitory effect of the NMDAR antagonist APV (Kew and Kemp 2005) was also tested. APV was applied for 10 min in 25 μM concentration according to the described electrophysiological recording protocol. The slices of the normal group displayed no alteration of relative EFP amplitude in the presence of APV (Fig. 3a). However, APV caused a mild suppression of the evoked response in the seizured group between 2T and 3T intensities (Fig. 3a).

The inhibitory effect of APV was also investigated in both groups at 2T stimulus intensity. In normal group, the relative amplitude of the EFP showed no change (Fig. 3b). At the end of the 10 min incubation period the relative amplitude of EFPs at 2T intensity was 102.0 ± 7.2 % in control slices (Fig. 3c; $n = 7$) while 97.0 ± 6.0 % was measured in the presence of APV (Fig. 3c; $n = 5$, $p = 0.627$, compared to control). Although in the seizured group significant inhibitory effect of APV on the relative amplitude of the N1 component was observed, the change was less enhanced (Fig. 3d). The relative amplitude of EFP at 2T stimulus intensity was 107.9 ± 6.4 % (Fig. 3e; $n = 6$) in control slices at 10 min, while 84.9 ± 5.1 % (Fig. 3e; $n = 6$, *$p = 0.019$, compared to control) was observed in APV-treated slices.

According to the statistical analysis, there was no difference in the relative amplitude of EFP in control slices of the normal and the seizured group ($p = 0.562$). Similarly, no differences were detected between the slices of normal and seizured groups ($p = 0.156$) following APV treatment, suggesting negligible inhibitory efficacy of APV in entorhinal cortex slices of both tested groups.

4-AP Treatment Reduced Co^{2+} Uptake Via Non-NMDARs

Kainate induces Co^{2+}-uptake through the activation of Ca-permeable AMPARs and KARs. Dark Co^{2+} precipitate formed with $(NH_4)_2S$ can be detected and evaluated with image analysis (Borbely et al. 2009; Vilagi et al. 2009). The difference in the optical densities (OD) between background and kainate-treated slices was used to estimate the abundance of Ca^{2+}-permeable AMPARs/KARs. Repeated 4-AP treatment caused a significant decrease in Co^{2+}-uptake in every layer of the entorhinal cortex (Fig. 4a, b). In slices of normal rats, the density of Co^{2+} staining was high in the outer layers I–III, and decreased

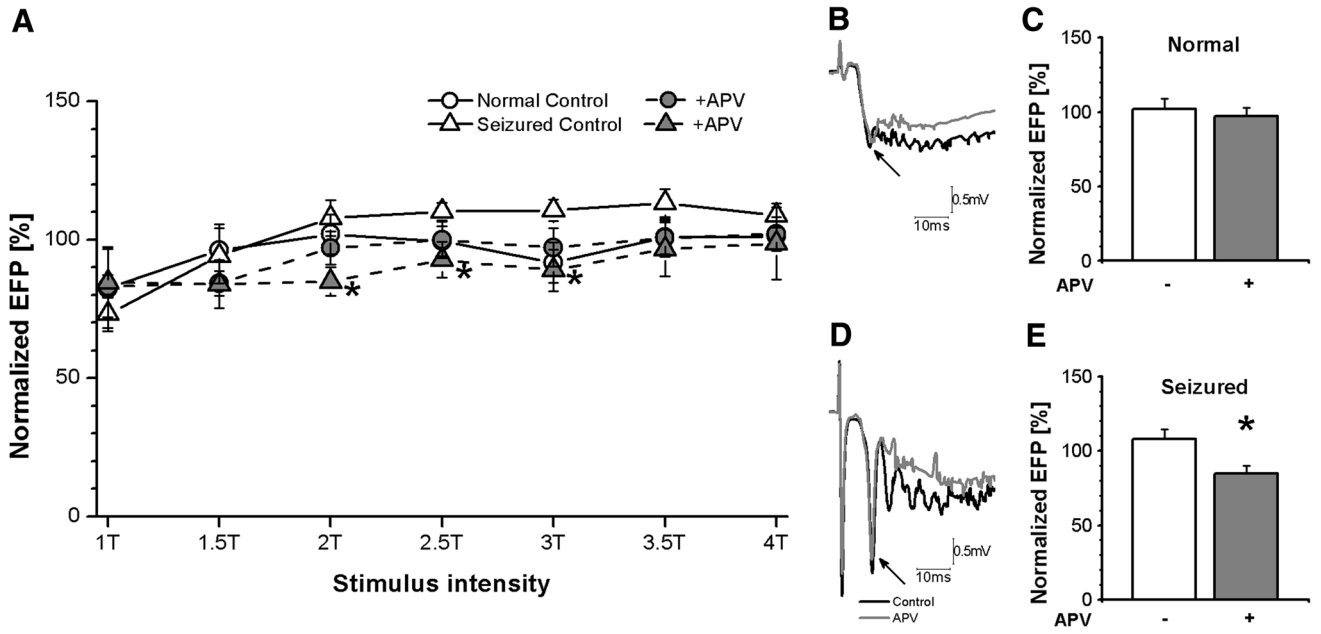

Fig. 3 The effect of 4-AP treatment on APV inhibitory efficacy. **a** Relative EFP value of N1 component was plotted against stimulus intensity showing the effect of the NMDAR antagonist APV (25 μM) in slices from normal and seizured animals. APV had no effect in slices obtained from normal rats at any intensity, while moderate inhibitory effect was observed in slices of seizured group (*$p < 0.05$). **b** Characteristic EFP traces of APV effect in normal group. The *arrow* indicates the N1 component of EFP. **c** Antagonistic effect of APV on EFP induced by 2T stimulus intensity in slices of normal group. The relative N1 amplitude showed no change in the presence of APV ($n = 5$, $p = 0.627$). **d** Characteristic EFP traces of APV effect in seizured group. The ↑ indicates the N1 component of EFP. **e** Antagonistic effect of APV on EFP induced by 2T stimulus intensity in slices of seizured group. The relative N1 amplitude due to the APV was significantly reduced ($n = 6$, *$p = 0.019$). Despite the mild inhibition of EFP in slices of seizure group, the efficacy of APV did not differ in seizured group compared to normal group ($p = 0.47$)

gradually towards the inner layers (IV–VI). In the seizured group, the level of Co^{2+}-uptake was significantly lower in layers I–III without detectable changes in deeper layers (IV–VI; Fig. 4b), compared to normal group. In the seizured group, 90.0 ± 3.9 % reduction of Co^{2+}-uptake was observed, indicating a significant reduction in the density of functional Ca^{2+}-permeable AMPAR/KARs.

4-AP-Induced Changes in iGluR Subunit Protein Levels

The 12-day-long daily 4-AP treatment caused differential changes in the expression of iGluRs subunits. The subunit expression levels were determined in 20 predefined regions of interest, located in the entire thickness of the entorhinal cortex, from the pial surface to the border of layer VI at the white matter. Here we indicated the ΔOD values in cortical layer III (at 810 μm cortical depth), where the recording electrodes were positioned during the electrophysiological experiments.

AMPAR/KAR subunit proteins

The overall levels of AMPAR subunit proteins (GluA1–4) were detected with a polyclonal antibody recognising the conserved extracellular loop region of all four AMPAR subunits (GluA1–4, both *flip* and *flop* splice variants; (Pickard et al. 2000)). Reduced GluA1–4 expression was detected in all layers of the entorhinal cortex in samples obtained from seizured rats (in layer III: 71.8 ± 1.4 ΔOD for control and 62.8 ± 1.6 ΔOD for seizure group, ***$p = 0.0005$, normal $n = 6$ rat; seizured $n = 7$ rat; Fig. 5, GluA1–4). To identify differential changes in individual AMPAR subunit proteins, experiments were performed with specific antibodies against GluA1 or GluA2 AMPAR subunits. While the GluA1 subunit expression was increased in supragranular layers (in layer III: 14.1 ± 0.5 ΔOD for control and 15.7 ± 0.6 ΔOD for seizure group, *$p = 0.048$, normal $n = 6$ rat; seizured $n = 7$ rat; Fig. 5, GluA1), the expression of GluA2 subunit was reduced in every cortical layer (in layer III: 20.7 ± 0.7 ΔOD for control and 17.1 ± 0.6 ΔOD for seizure group ***$p = 0.0007$, normal $n = 5$ rat; seizured $n = 7$ rat; Fig. 5, GluA2) in brain samples from seizured rats. Due to the lack of specific antibodies against GluA3, the expression level of this subunit protein was not tested. The density of GluA4 is very low in adult neocortex (Molnár 2008), therefore this subunit was not investigated in histoblot experiments. The expression level of GluK5 KAR

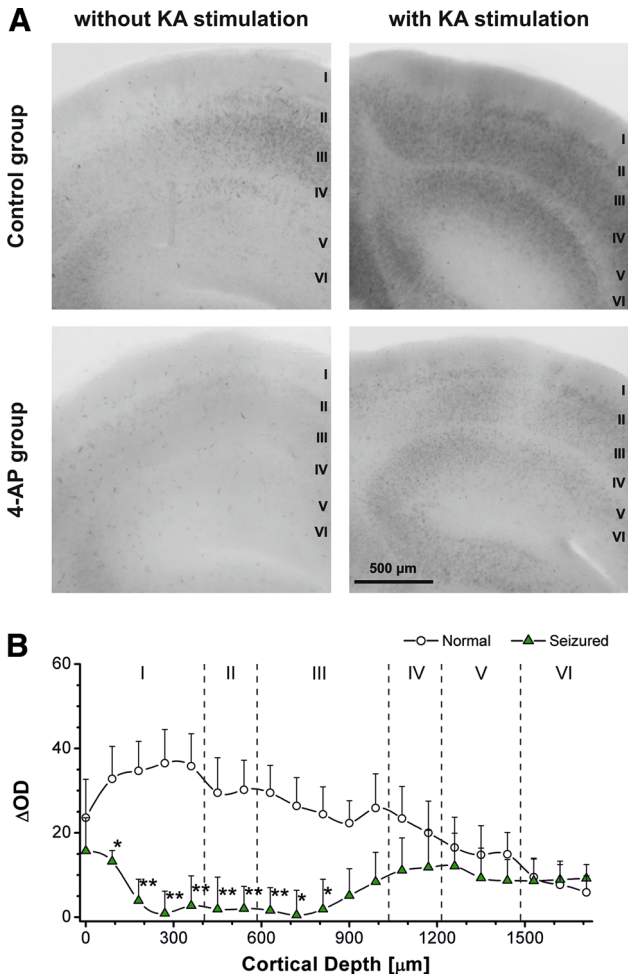

Fig. 4 Assessment of Ca^{2+} permeability of non-NMDARs. The *plots* represent kainate (KA)-stimulated Co^{2+}-accumulation in entorhinal cortical slices from normal and seizured rats. This method was used to estimate changes in Ca^{2+}-permeability of AMPARs/KARs in brain samples of seizured rats. **a** Characteristic images of brain slices obtained from normal and seizured rats without and with kainate stimulation. **b** Quantitative analysis of accumulated Co^{2+} based on determination of optical density indicates that 4-AP treatment evoked a significant reduction in Co^{2+}-uptake in cortical layers I–III (normal group: $n = 9$, seizured group: $n = 10$, $*p < 0.05$, $**p < 0.01$)

subunit decreased in all layers of the entorhinal cortex (in layer III: 23.1 ± 0.8 ΔOD for control and 16.4 ± 1.1 ΔOD for seizure group, $***p = 0.000004$, normal $n = 6$ rat; seizured $n = 6$ rat; Fig. 5, GluK5) on histoblots prepared from seizured rats.

NMDAR Subunit Proteins

The GluN1 subunit protein expression level was also investigated by the histoblot method. GluN1 subunit showed regional differences; the expression level was higher in layer I–III, than in layer IV–VI. On the other hand, no significant change was observed in correlation with 4-AP-induced seizures (in layer III: 62.0 ± 3.1 ΔOD for control and 52.8 ± 3.4 ΔOD for seizure group, $p = 0.16$, normal $n = 4$ rat; seizured $n = 6$ rat; Fig. 5, GluN1). In contrast, there was an increase in GluN2A expression in layer I and IV–VI of seizured rat brain samples. The layer III showed no significant change of GluN2A subunit (in layer III: 58.1 ± 3.7 ΔOD for control and 62.4 ± 1.7 ΔOD for seizure group, $p = 0.26$, normal $n = 4$ rat; seizured $n = 6$ rat; Fig. 5, GluN2A).

In summary, the daily 4-AP-induced seizure activity had differential and region-specific effects on iGluR subunit expression levels, causing an (i) overall reduction in GluA1–4 and GluA2 with a moderate increase in GluA1 AMPARs, (ii) marked reduction in GluK5 KARs, (iii) no significant change in GluN1 with an increase in GluN2A NMDAR protein levels.

Discussion

Our study revealed that repeated 4-AP-induced seizures elicit changes in responsiveness of the lateral entorhinal cortex, parallel with significant alterations of the appearance of iGluRs. The excitability of the entorhinal cortex slices obtained from the seizured rats was enhanced compared to sham-treated normal animals. While the AMPAR antagonist GYKI 52466 exerted a reduced inhibitory effect on evoked EFPs in brain slices of seizured group, the effects of the NMDAR antagonist APV mildly increased. Kainate-induced Co^{2+}-uptake was significantly reduced in slices from the seizured rats. Histoblot analysis revealed an overall decrease in the density of AMPARs after repeated 4-AP treatment with a moderate increase in the density of GluA1 subunits and a significant decrease in the density of GluA2 subunits. There was also a substantial reduction in GluK5 receptor subunit levels in all cortical layers. As to NMDAR's, there was a weak increase in GluN2A subunit levels without significant change in GluN1 following repeated daily seizures.

4-AP is a widely used convulsant in vivo as well as ex vivo, in brain slice experiments, thus its effects are well characterised (Borbely et al. 2009; Kopniczky et al. 2005; Pena and Tapia 2000; Vilagi et al. 2009). Systemic intraperitoneal application of 4-AP evokes general depolarisation and excessive transmitter release via blockade of K^+ channels and leads to the development of immediate seizures (Kovacs et al. 2003; Mihaly et al. 1990; Thesleff 1980). The administration of 4-AP elicits generalised tonic–clonic seizures in vivo in rats (Mihaly et al. 1990) and seizure-like discharges in brain slices (Doczi et al. 1999). The 4-APinduced seizure activity is regarded as a conventional model of the generalised seizure (Avoli et al. 1988; Pena et al. 2002; Traub et al. 1996). Unlike other convulsants such as kainate (Nadler et al. 1978) or

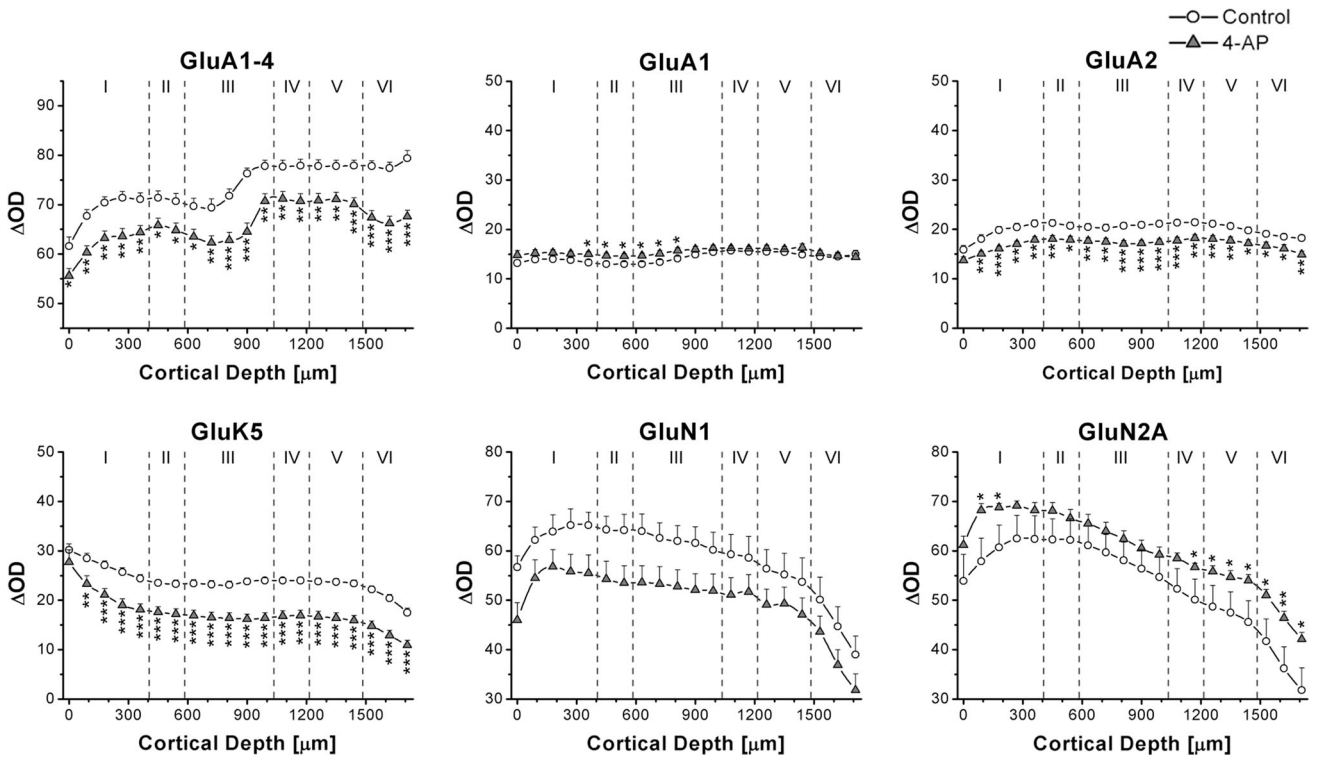

Fig. 5 Changes in iGluR subunit protein expression levels following repeated 4-AP treatment. Quantitative densitometry of AMPAR, KAR and NMDAR subunit immunoreactivities identified differential changes in iGluR subunit proteins. The level of total AMPARs (GluA1–4) decreased significantly in all layers except layer II. GluA1 subunit levels moderately increased mainly in layer II and adjacent layers. In contrast, GluA2 subunit levels significantly decreased in all cortical layers. The level of GluK5 KAR subunit decreased in all cortical layers. The expression of GluN1 showed no change due to 4-AP treatment, while GluN2A exhibited upregulation in layers I–III. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

pilocarpine (Cavalheiro et al. 1991), which cause excessive cell damage, 4-AP treatment does not lead to chronic epilepsy. Rats may develop some tolerance towards 4-AP (Borbely et al. 2009; Vizi et al. 2004).

AMPARs/KARs play a crucial role in neuronal excitability and their subunit composition influences the synaptic efficacy and also the seizure susceptibility of a cortical network (Jane et al. 2009). It was proved in patch clamp experiments that characteristics of spontaneous excitatory postsynaptic current (sEPSCs) of inhibitory and excitatory cortical neurons are different, which depend on their dissimilar AMPAR/KAR composition. Non-NMDA receptors of aspiny inhibitory neurons are more sensitive to inhibition (Hestrin 1993). The GluA2 subunit has a very important role in determining the functional characteristics of AMPARs. The presence of the Q/R-edited GluA2 subunit makes AMPARs impermeable to Ca^{2+}, which results in a decrease of receptor conductance and linear I–V characteristics (Isaac et al. 2007; Traynelis et al. 2010). In the adult brain, nearly all native AMPARs contain the edited form of the GluA2 subunit (Appleby et al. 2011; Michaelis 1998; Molnar and Isaac 2002), which protect the neurons from the serious Ca^{2+} toxicity and overactivation. The expression of GluA2 is strictly regulated; its sustained downregulation may lead to neurodegeneration by enhancing Ca^{2+} influx (Pellegrini-Giampietro et al. 1997). In previous studies, altered subunit composition was reported in the hippocampus, following entorhinal cortex lesion (Pellegrini-Giampietro et al. 1997). Our recent findings revealed the decreased expression of GluA2 in all entorhinal cortical layers following repeated seizures. On the other hand, the overall decrease of the AMPAR subunits and the reduction of GluK5 expression may diminish the expected enhancement of Ca^{2+} influx. The reduced kainate-activated Co^{2+}-uptake suggests that the reduction in GluA2 levels was antagonised by GluK5 and GluA1–4 downregulation. The proportional change in GluK5 expression was twofold compared to GluA2. Since the Ca^{2+} permeability of KARs is higher than that of the AMPARs (Stricker and Huganir 2002), the downregulation of the GluK5 subunit, (with parallel reduction in AMPAR levels) caused the decrease of neuronal Ca^{2+}-permeability. Our preceding experimental results obtained in other brain regions support this hypothesis: in the somatosensory cortex, the reduced expression of AMPAR subunits without changes in GluA2 and GluK5 expression efficiently

decreased the Ca^{2+} permeability (Vilagi et al. 2009). On the other hand, the CA1 region of the hippocampus displayed the decrease of GluA2, together with unchanged GluA1–4 and GluK5, but the net result was a significant increase in Ca^{2+} permeability (Borbely et al. 2009). It has been demonstrated that Co^{2+}-uptake do not provide information about the overall Ca^{2+} intake, i.e. the permeability changes of NMDARs, because these types of receptors are impermeable to Co^{2+} (Mayer and Westbrook 1987).

It was reported that other convulsants also cause alterations in receptor number and subunit composition of AMPARs (Pellegrini-Giampietro et al. 1997; Tolner et al. 2007). The common feature of this phenomenon was the increased Ca^{2+} permeability of AMPARs due to GluA2 downregulation (Rajasekaran et al. 2012). Rearrangement of GluA1–4 subunit expression, parallel with a reduced GluA2 subunit manifestation was observed in the CA3 region of rat hippocampus, following kainate-induced status epilepticus (Friedman et al. 1994; Friedman and Veliskova 1998). Similar observations were made in pilocarpine-induced seizures (Lason et al. 1997; Rajasekaran et al. 2012). On the other hand, in early postnatal seizures induced by pilocarpine, in the rat hippocampus an elevated GluA1–3 subunits level and unchanged GluA4 was observed (Silva et al. 2005). Downregulation of GluA2 can be also induced in rat hippocampal CA1 region by transient forebrain ischemia (Pellegrini-Giampietro et al. 1997), in layer V neocortical pyramidal neurons by in vivo cortical lesion (Kharazia and Prince 2001) and in cortical neurons by kainate administration (Jia et al. 2006). The chronic treatment with pentylenetetrazole did not result in significant overall reduction of AMPARs. In these experiments, however, a significant decrease of KARs was reported (Cremer et al. 2009), and the level of GluA2 subunit expression was reduced by 50 % (Cremer et al. 2009). Similarly, we also detected a significant 15 % reduction of GluA2 subunit level.

GYKI 52466 is a non-competitive inhibitor binding to an allosteric site of AMPARs (Tarnawa et al. 1990). Therefore, its inhibitory effect is independent from glutamate binding or extracellular glutamate levels (Wilding and Huettner 1995). Therefore, the most likely explanation of 4-AP treatment-related reduction in GYKI 52466-induced inhibition is that AMPAR/KAR levels and/or subunit composition altered as a consequence of 4-AP provoked seizures. GYKI 52466 has no preference to *flip* or *flop* isoforms of AMPARs (Johansen et al. 1995), but changes of the subunit composition may influence GYKI 52466 antagonism. GYKI 52466 is a less effective inhibitor on homotetrameric receptors formed by GluA1 or GluA4 subunits than on heterotetramers of GluA1/GluA2 or GluA2/GluA4. This suggests that the presence of GluA2 subunits in AMPARs increases the antagonistic effect (Bleakman et al. 1996; Johansen et al. 1995). Since GYKI 52466 specifically inhibits AMPARs in the applied concentration (Bleakman et al. 1996), we can state that the decrease of the GYKI 52466 effect was related to the overall decrease of AMPARs and the decrease of the GluA2 subunit expression.

Despite the fact that the expression of NMDAR subunits is strictly regulated (Lujan et al. 2005), the alteration of subunit composition can be induced by different convulsants. The available experimental findings are, however, controversial. Perinatal kainate-induced seizures result in upregulation of GluN2A and GluN2B subunits (Gashi et al. 2007), while their downregulation was observed after repeated pilocarpine-induced status epilepticus (Silva et al. 2005) in the rat hippocampus. In adult rats pentylenetetrazole-kindling reduced the expression of GluN2A without changes in GluN1 and GluN2B levels (Zhu et al. 2004), suggesting that GluN2A downregulation was necessary for the increase of general excitability. Experimental data from human patients with focal cortical dysplasia suggest that the upregulation of NMDAR subunits is necessary for increased epileptogenesis (Najm et al. 2000). We have detected a mild upregulation of GluN2A subunit which may underlie the parallel reinforce of APV inhibitory effect.

The number, molecular composition and functional properties of receptors fundamentally influence the characteristics of evoked field potentials in brain slices. In this study, we identified an enhanced responsibility of the entorhinal cortex following repeated 4-AP-induced seizures. Nevertheless, this was accompanied by an overall reduction in the expression levels, Ca^{2+} permeability and antagonist sensitivity of AMPARs and KARs. However, in parallel a moderate but detectable GluN2A receptor expression increase was obtained. Our previous findings revealed similar alterations of iGluR structure in the somatosensory cortex (Vilagi et al. 2009), and in the CA3 region of the hippocampus (Borbely et al. 2009) of seizured rats. But the general excitability changes detected in ex vivo slice preparations were not uniform: we measured decreased excitability in the somatosensory cortex (Vilagi et al. 2009), and increased excitability in the hippocampus (Borbely et al. 2009) and entorhinal cortex. The shape and the amplitude of the evoked field response in slices from different cortical regions are basically determined by the activation of excitatory and inhibitory neurons in the local networks. The overall excitability depends on the balance of the activity of excitatory and inhibitory cells, which strongly depends on the actual receptor pool expressed. The inhibitory interneurons also possess excitatory amino acid receptors. As these types of neurons are more prone to overexcitation, so following the seizures the number of them may decrease, parallel decreasing the whole inhibition. So diminish of the

whole ionotropic excitatory amino acid receptors may result in a net increase of EFP (Hestrin 1993). According to our studies, long lasting 4-AP treatment mostly results in alterations in non-NMDARs, the number of which may decrease in all cell types. As a consequence, inhibition will be less efficient in that brain area, which may alter the local network activity, together with the regional sensitivity. Simultaneously, the moderately enhanced NMDAR activity may explain an overall increase in excitability. We hypothesised, that non-NMDAR-mediated processes are less sensitive, so no spontaneous seizure activity develops following the repetitive 4-AP application. Further investigations are needed to define the prominently affected cell types, synapses and networks.

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Conflict of interest The authors declare that they have no conflict of interests.

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