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Blockade of AMPA-receptors attenuates 4-aminopyridine seizures, decreases the activation of inhibitory neurons but is ineffective against seizure-related astrocytic swelling

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Summary The neurotransmitter glutamate plays a pivotal role in the development of the neuropathological sequelae following acute seizures. Our previous data proved the efficacy of the NMDA-receptor antagonists on the symptoms, survival and neuronal activation in the 4-aminopyridine- (4-AP) induced seizures. In this study, we examined the effects of two different doses of a non-competitive, selective, allosteric AMPA-receptor antagonist, GYKI 52466. GYKI 52466 was effective in prolonging the latency to generalised seizures and reduction of seizure mortality. However, the effects on neuronal c-fos expression and astrocyte swelling were complex. The 25 mg/kg dose of GYKI 52466 was effective in reducing the c-fos immunoreactivity (IR) in the hippocampus only. In the neocortex the overall c-fos-IR cell counts were increased significantly. Investigation of the neocortical parvalbumin-containing interneuron population proved that GYKI 52466 decreased c-fos expression. The 50 mg/kg dose of GYKI 52466 significantly reduced the c-fos-IR in the neo- and allocortex, not only in principal neurons, but also in the parvalbumin-positive interneurons. The GYKI 52466-pretreatment did not prevent the astrocyte swelling in the investigated cortical areas; thus we conclude that the AMPA-receptors have little if any involvement in the in the mediation of neuropathological alterations in acute convulsions.

1. Introduction

The excessive, pathologic oversynchronisation (Perrault and Avoli, 1991; Yang and Benardo, 2002) of neuronal activity and the disequilibrium between certain excitatory and inhibitory factors (Gulyás-Kovács et al., 2002; Schwaller

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et al., 2004) are considered as the pathophysiologic basis of the epileptiform activity in the central nervous system. The conventional therapeutic regimes concentrate on the strengthening of the inhibitory neurotransmission (MacDonald et al., 1985; Lukasiuk and Pitkänen, 2000) whereas the inhibition of the excitatory responses is under current research, but results are mainly available about the role of NMDA-mediated events (Conti and Weinberg, 1999; Borowicz et al., 2001; Szakács et al., 2003).

One of the well-known (Mihály et al., 1990; Peña and Tapia, 2000) chemical means to elicit acute seizures with generalised tonic-clonic features in an *in vivo* animal model is the 4-aminopyridine (4-AP), having well-circumscribed pharmacokinetic (Lemeignan et al., 1984; Berger et al., 1989) and pharmacodynamic (Perrault and Avoli, 1991; Mihály et al., 2000) properties. 4-AP is a nitrogen-containing heterocyclic K^+-channel blocker, exerting its effect mainly via IK(A) and IK(V) channel types (Brückner and Heinemann, 2000), thus the shift of the membrane potential towards depolarisation enables Ca^{2+}-influx; mainly via voltage-gated Ca^{2+}-channels and the NMDA-receptor ion channels (Greenberg and Ziff, 2001), the latter are opened by the mainly presynaptically acting 4-AP-induced membrane-depolarisation (Yang and Benardo, 2002). The sustained, repeated, synchronised neuronal discharge, the so-called ''burst firing'' is a basic feature of the central nervous system seizure mechanism (Labiner et al., 1993). The prolonged neuronal depolarisation stimulates both excitatory and inhibitory neurotransmitter release (Versteeg et al., 1995), especially glutamate (Peña and Tapia, 1999; Kovács et al., 2003) and reinforces the inhibitory (IPSPs) and excitatory postsynaptic potentials (EPSPs) (Perrault and Avoli, 1991). On the basis of the electrophysiological investigations (Yang and Benardo, 2002), one of the 4-AP-induced cortical spontaneous discharge components can be blocked by glutamate receptor antagonists, whereas the other type by $GABA_A$ receptor antagonists, suggesting that it was produced by the synchronisation of GABAergic (GABA, γ-amino-butyric acid) interneurons.

The immediate early gene (IEG) c-fos (Morgan and Curran, 1991) is an inducible transcription factor (ITF) playing important role in certain nuclear regulative processes (Dragunow et al., 1989). The gene products of the IEGs, via transcriptional regulation influence the genes involved in the maturation and adaptation mechanisms of the nervous system (Retchkiman et al., 1996; Schmoll et al., 2001), or in plasticity and neurodegeneration (Herdegen and Waetzig, 2001), as well as in epilepsy (Gass et al., 1992; Rocha et al., 1999). The c-fos mRNA transcription shows strict correlation with the electrophysiologically proven cellular activity (Labiner et al., 1993), and this correspondence establishes the Fos protein (appearing in the cell nuclei of the neurons concerned) as a sensitive marker of the increase of neuronal activity (Hoffmann and Lyo, 2002). NMDA- and AMPA-receptors are amongst the external signals, which activate c-fos transcription (Herdegen and Leah, 1998).

Amongst the earliest neuropathological changes in seizure activity (Kelso and Cock, 2004), the swelling of the astrocytic processes has already been observed (Fujikawa et al., 1992; Fabene et al., 2006). Astrocytes are the main cell types that swell in cytotoxic brain oedema (Kimelberg, 2004), especially the pericapillary foot processes, which are the predominant sites of aquaporin-4 (AQP4) expression in the brain (Papadopoulos and Verkman, 2007). Glutamate, at similar concentrations required to induce neuronal cell death, has been shown to increase cell volume in cultured astrocytes (Han et al., 2004). The astrocytic swelling has numerous deleterious secondary effects, such as the release and decreased uptake of excitatory amino acids (Kimelberg, 2004); worsening the micro-environmental circumstances like a *circulus vitiosus* process. Pharmacological inhibition of seizure activity decreases brain oedema in different animal models (Clifford et al., 1990; Tian et al., 2005).

The GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride) is a selective, non-competitive, presumably allosteric antagonist of the AMPA-(α-amino-3-hydroxy-5-methyl-4-isoxazol propionate)-subtype ionotropic glutamate receptors (Donevan and Rogawski, 1998; Arai, 2001); with good blood—brain-barrier (BBB) permeability (Barna et al., 2000), neuroprotective effects in seizure and cerebral ischaemia (Szabados et al., 2001; Arias et al., 1999), antinociceptive and anti-inflammatory properties (Székely et al., 1997; Szabados et al., 2001) and has other advantageous effects in ischaemic conditions (Block et al., 1996; Arias et al., 1999). Electrophysiological investigations (Paternain et al., 1995; de Sarro et al., 1995) proved the antagonistic activity of GYKI 52466 upon the AMPA- and kainate-receptors, whereas practically no effect has been demonstrated on the NMDA-receptors, metabotropic glutamate receptors, and on the $GABA_A$-receptors. According to the kinetic analysis of AMPA-receptors, the most likely way of GYKI 52466 effect is elongating the desensitisation—resensitisation process, the channel closing frequency and the deactivation (Arai, 2001).

In our former studies (Szakács et al., 2003) the NMDA-subtype ionotropic glutamate receptor antagonists in pretreatment significantly decreased the expression of c-fos in the examined neo- and allocortical areas in the 4-aminopyridine acute convulsive rat model; and in the same time, the symptoms of seizure were eliminated, as well. NMDA-antagonists significantly decreased seizure-related astrocyte swelling in the cerebral cortex (Zádor and Weiczner, unpublished). The aim of the present study was to assess the antagonism of another well-characterised (Alexander and Peters, 2000) glutamate receptor, the AMPA-receptor in a similar paradigm. According to the time-course of the GYKI 52466 anticonvulsive efficacy (Lees and Leong, 2001; Jakus et al., 2004), and based on our preliminary data we focused on the investigation of the short-term effects (c-fos expression and pericapillary astrocyte swelling) within the very first hour of seizure initiation. To evaluate the activation status changes of the inhibitory cells in the 4-AP paradigm, we also investigated the effect of the AMPA-receptor antagonism by assessing the c-fos expression pattern of the immunohistochemically well-detectable parvalbumin (PV)-positive cells (Schwaller et al., 2004) representing a subpopulation of GABAergic interneurons (DeFelipe, 1997).

2. Materials and methods

2.1. Animals and treatment

The animal experiments were conducted in accordance with prevailing laws and ethical considerations (European Community Council Directive of 24 November 1986 (86/609/EEC) and the Hungarian Animal Act (1998). Written permission was obtained in advance from the Faculty Ethical Committee on Animal Experiments (University of Szeged). The animals were maintained under standard animal housing conditions, with *ad libitum* access to food and water. The experiments were performed on male Wistar rats weighting 200—250 g. The convulsant agent 4-aminopyridine (4-AP; Sigma, St. Louis, MO) was dissolved in physiologic saline (0.67 mg in 1 ml vehicle) and administered intraperitoneally (i.p.), in 5 mg/kg dose. In previous investigations (Mihály et al., 1990), this dosage proved to be convulsant. The non-competitive AMPA-receptor antagonist GYKI 52466 (IVAX GYKI Co. Ltd., Budapest, Hungary) was dissolved in 50% DMSO (de Sarro et al., 1995) (dimethyl-sulphoxide; Sigma, St. Louis, MO) dilution (3.33 or 6.67 mg in 1 ml vehicle), and administered i.p. in 25 or 50 mg/kg dose, respectively.

2.2. Experimental procedure

The animals were randomly divided into three groups. In the first 2 groups, the animals (10 per group) were pretreated with 25 or 50 mg/kg GYKI 52466, respectively. 15 min later, the convulsant 5 mg/kg 4-AP was administered. The control group (10 animals) received the solvent of GYKI 52466 and 5 mg/kg 4-AP. The experiments were finished 1 h after the 4-AP injection, within the range of the presumed maximal anticonvulsive effect of the GYKI 52466 (de Sarro et al., 1998.). At the end of the experiments, the animals were deeply anaesthetised with diethyl-ether and perfused transcardially with 250 ml of 0.1 M phosphate-buffered saline (PBS) pH 7.4; followed by 300 ml of fixative (four animals randomly for immunohistochemistry: 4% phosphate-buffered paraformaldehyde, pH 7.4; four animals randomly for electron microscopy: 1% paraformaldehyde and 1% glutaraldehyde in phosphate-buffered solution, pH 7.4). In case of the brains for immunohistochemistry, they were rapidly removed, postfixed in 4% paraformaldehyde for 1 h, and then cryoprotected overnight (30% sucrose in 0.1 M phosphate buffer, pH 7.4) at room temperature. Serial frozen sections were cut on a cryostat (Reichert-Jung Cryocut 1800) in the coronal plane at a thickness of 24 μm and every third section was processed for immunohistochemistry (2.3). In case of the brains for electron microscopy, they were rapidly removed, and then we followed the routine procedure as described below in Section 2.4.

2.3. Immunohistochemistry

On the coronal brain slices c-fos and parvalbumin double-labelling immunohistochemistry was carried out. The sections were pretreated with Triton X-100 and H_2O_2 (9:1) solution, then they were incubated in 20% normal pig serum, avidin and biotin; then in the primary antibody cocktail with c-fos antibody (1:2000; raised in rabbit, Santa Cruz Biotechnology, CA) and parvalbumin (1:60,000; raised in mouse, Sigma—Aldrich). The secondary antibody cocktail contained biotinylated goat-anti-rabbit (B-GAR, 1:400; Jackson ImmunoResearch, PA) and goat-anti-mouse (GAM, 1:400; Jackson ImmunoResearch, PA) antibodies. The tertiary antibodies were STA-PER (1:1000, Jackson ImmunoResearch, PA) and mouse-PAP (1:1000, Jackson ImmunoResearch, PA). The peroxidase reactions were localised with diaminobenzidine tetrahydrochloride (Sigma—Aldrich) with nickel chloride, yielding black reaction product (c-fos) or without nickel chloride, yielding brown reaction product (parvalbumin).

2.4. Electron microscopy

Samples of the right parietal neocortex and hippocampus were prepared for electron microscopy. The tissue blocks were incubated in an aqueous solution of 1% OsO_4 and 5% $K_2Cr_4O_7$ (1:1) after thorough rinsing. The samples were dehydrated, incubated in 1% uranyl-acetate and embedded in Durcupan epoxy resin (Fluka, Buchs, Switzerland). Semithin sections were cut on an ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria) and stained on object glasses with a 1:1 mixture of 1% methylene blue and 1% Azure II blue. The samples were then coverslipped with DPX and analysed under a light microscope (Nikon E600, Nikon Co., Tokyo, Japan). Ultrathin sections were cut of the same blocks and collected on 200 mesh copper grids. The preparations were then contrasted with 5% uranyl-acetate and Reynolds lead-citrate solution. Finally, the samples were analysed by a Philips TM10 transmission electron microscope (Eindhoven, Netherlands). Photographs were taken with a computer assisted digital camera (MegaView II, Soft Imaging Systems, Münster, Germany).

2.5. Analysis of the behavioural data

The behavioural outcome (effects concerning seizure activity and possible adverse effects) of the pretreatment with the AMPA-antagonist was observed up to 1 h after the 4-AP injection. The onset of the GTCS was always sudden and clear-cut, so the latency (from the 4-AP injection) of GTCS was easily measurable. The GTCS latency was statistically evaluated with one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test (significance criterion was 0.05). The GTCS occurrence and overall mortality data were analysed with the Fisher's exact probability test (significance criterion was 0.05). The statistical analysis was performed with the SPSS 9.0 software.

2.6. Analysis of the immunohistochemical data

Quantitative analysis was performed on five sections per animal ($N = 4$; the mean data per each animal was used in the statistical analysis), selected from every brain on the basis of the same stereotaxic coordinates (Paxinos and Watson, 1998). Areas of interests (AOIs) for counts of immunostained neuronal nuclei were selected form the S1Tr region of the parietal neocortex, regions CA1, CA2 and CA3 of the Ammon's horn and from the hilus and granule cell layer of the dentate gyrus. Within every AOI, the immunoreactive (IR) cells (cell nuclei for c-fos and cells for PV) were counted using a NIKON Eclipse 600 microscope equipped with a SPOT RT Slider digital camera (1600 × 1200 dpi in 8 bits), using the Image Pro Plus 4 morphometric software (Media Cybernetics, Silver Spring, MD). Following background subtraction, the threshold was determined so that all labelled nuclei could be recognised. The counting was performed blindly of the animal's treatment. The AOIs were determined using the rectangular field of the camera. In the neocortex, cell counts were done using a 10× objective, and the AOI (an area of 1.2 mm^2) included all neocortical layers (I-VI) from the pia mater to the subcortical white matter. Cell counts were then normalised to 1 mm^2. In the hippocampus, cell counts were done using a 40× objective, and were again normalised to 1 mm^2. In regions CA1-3, the AOI (an area of 0.05 mm^2) included the stratum pyramidale and a narrow zone of the strata oriens and radiatum. The hilus of the dentate gyrus was outlined according to Amaral (1978), and counting was performed. The whole extent of the upper and lower blades of the dentate granule cell layer was outlined and used as AOI, and labelled cell nuclei were counted in this area. The quantitative assessment of the parvalbumin-positive cells was omitted in the granular layer of the dentate gyrus, due to the many, strongly c-fos immunoreactive cell nuclei. The data were analysed statistically comparing sets of findings with the same magnification. Differences

in the number of c-fos positive or c-fos and parvalbumin double-positive cells in the control and in the antagonist-pretreated were analysed with one-way analysis of variance (ANOVA), followed by the Bonferroni *post hoc* test. A significance criterion of 0.05 was used. The statistical analysis was performed with the SPSS 9.0 software.

2.7. Analysis of the electron microscopical data

Approximately 900 μm^2 of sample surface was viewed systematically through all neocortical layers of the parietal cortex or in the hippocampus; four EM preparates per animal ($N = 4$) was examined and the mean data of the altogether 14 ± 2 capillary cross sections per each animal was used in the statistical analysis. The total area of the so-called neurovascular unit was measured which consists of the capillary lumen, the surrounding endothelium and the astrocytic end-feet covering the basal lamina of the microvascular endothelium. (Image Pro Plus 4.5 morphometric software; Media Cybernetics, Silver Spring, MD, USA). One-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test (significance criterion: $p < 0.05$; SPSS 9.0 statistical software) was performed on the measured area data (area of capillaries, area of the swollen pericapillary astrocytic endfeet).

3. Results

3.1. Behavioural analysis

The i.p. administration of 4-AP causes characteristic behavioural symptoms within 15—20 min (Mihály et al., 1990): increased exploratory activity, tremor of the vibrissal and masticatory muscles, followed by generalised tremor of the body musculature, observable as continuous fasciculation of the muscles, the more and more frequent clonuses and tonus changes of the limbs. Abduction of the posterior limbs and a fan-like abduction of the fingers are also observable. The increasing motor symptoms lead to a ''wild running'' phenomenon and, finally, to generalised tonic-clonic seizures (GTCS), often preceded by vocalisation. The limbs are extended; hypersalivation and release of excreta are regular. With the dissipation of the tonic component, the clonic one becomes more dominant for a while with tenebrosity (post-convulsive state with limited cognitive and motor activity) for other 10—15 min until regaining normal activity. In some cases the GTCS may occur once more. The gradual dissipation of the seizure activity can be detected electrophysiologically after 50—60 min (Mihály et al., 2005); 90—100 min after the 4-AP injection, the animals recover. Nevertheless, approximately 20% of the control group animals die during or after GTCS, due to the seizure. The onset of the GTCS was always sudden and clear-cut, so the latency (from the 4-AP injection) of GTCS was easily measurable.

The increase in the GTCS latencies of the GYKI 52466-pretreated groups is observable but statistically do not differ between the two dose groups, however, they represent a significant change compared with the control group (Table 1). In the pretreated groups, the GTCS occurred in 20% (25 mg/kg) or in 10% (50 mg/kg) of the animals, whilst in 80% of the 4-AP control animals (without GYKI 52466 pretreatment). According to the Fisher's exact probability test, reduction in the number of animals (in which a GTCS occurred) in both groups administered GYKI was statistically significantly different from the number in the vehicle treated group. Nevertheless, there is no significant difference between the groups with different GYKI 52466 doses. During the 1-h observation, no recurrent GTCS was observed, the survival ratio was 100% in both pretreated groups, whilst 80% in the control group; which is, however, statistically not significant. Besides the local — presumably peritoneal — irritation (at the site of injection) dissipating within 1—2 min after the DMSO administration (as vehicle in the control group), no long-lasting side-effect of DMSO was noted, the animals displayed normal activity. As observable side-effects of the GYKI 52466 pretreatment, transient (approximately 15—20 min before regaining normal activity) ataxia, loss of coordination and reduction of the locomotor activity, together with apparently sedative effect (decreased vigilance) were noted (i.e. the animals stopped the explorative movements, laid down at the floor of the cage, breathing evenly similar to sleeping, their overall muscle tone appeared to be reduced at holding or palpating the animals). These changes were pure visual observations; no methodical evaluation was performed on these parameters.

3.2. Immunohistochemistry

The lower dose GYKI 52466 pretreatment (25 mg/kg) significantly increased, whereas the higher dose pretreatment (50 mg/kg) significantly decreased the number of c-fos-IR cell nuclei in the neocortex (Fig. 1). In laminar distribution, the laminae II—III and V—VI showed this significant decrease with the higher dose (the decrease in the lamina IV was not significant). The lower dose pretreatment yielded significant increase only in the lamina V, the other changes are statistically not significant. In the lamina I, no change was detectable in both cases. As for the c-fos and PV

Table 1 Behavioural analysis of the effect of the AMPA-receptor antagonism on 4-AP seizures

Pretreatment	Treatment	Mean GTCS latency (min)	S.E.M.	Animals displaying GTCS (%)	Mortality (%)
vehicle of GYKI 52466 i.p.	5 mg/bw kg 4-AP i.p.	26.60	1.81	80	20
25 mg/bw kg GYKI 52466 i.p.	5 mg/bw kg 4-AP i.p.	49.57*	2.31	20▲	0
50 mg/bw kg GYKI 52466 i.p.	5 mg/bw kg 4-AP i.p.	46.75*	4.21	10▲	0

The observation was made with 10 animals in each group. The GTCS (i.e. generalised tonic-clonic seizure) latency was measured from the administration time of the 4-AP injection. Mean GTCS latency: asterisk indicates the significant difference vs. control group (vehicle + 4-AP). *$p < 0.05$ ANOVA followed by the Bonferroni *post hoc* test. Seizure occurrence and mortality: black triangle indicates the significant difference vs. control group (vehicle + 4-AP). ▲$p < 0.05$ Fisher's exact probability test.

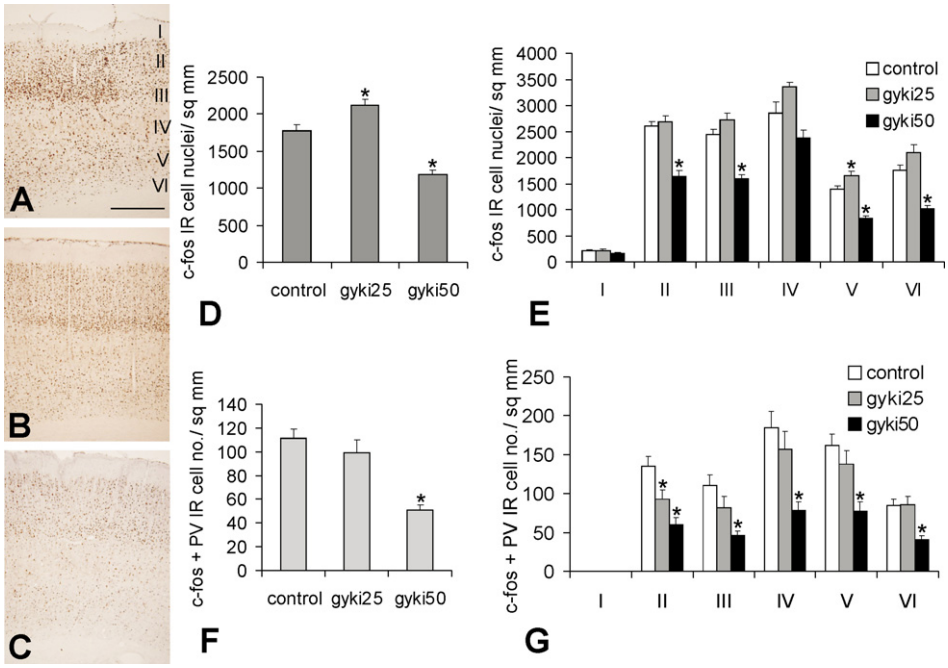

Fig. 1 Effect of GYKI 52466 on the c-fos gene expression in the neocortex. (A—C) Representative immunohistochemical images illustrating the c-fos and c-fos + parvalbumin (PV) immunolabelled neurons in the neocortex of the experimental animals. Animals with vehicle + 5 mg/kg 4-AP (control) (A); 25 mg/kg GYKI 52466 + 5 mg/kg 4-AP (gyki25) (B) and 50 mg/kg GYKI 52466 + 5 mg/kg 4-AP (gyki50) (C) treatment, respectively ($N = 4$ per group). Roman numerals indicate the neocortical laminae. Bar: 250 μm. Magnification: 40×. (D—G) Statistical evaluation of the c-fos (D—E) and c-fos and parvalbumin (F—G) immunoreactive cell counts pro square millimetre (sq mm), in all layers (D and F) and in laminar distribution (E and G); comparison of the data from pretreated (with 25 or 50 mg/kg GYKI 52466 before 5 mg/kg 4-AP, respectively) and the control animals (vehicle + 5 mg/kg 4-AP), 1 h after the seizure induction. Asterisks denote the significant differences (with vs. without GYKI 52466 pretreatment); (ANOVA, Bonferroni *post hoc* test, $p < 0.05$; S.E.M. is indicated in every case).

double-positive cells, the lower dose pretreatment had no effect while the higher dose pretreatment caused a significant decrease in the double-immunoreactive cell counts. This change was significant only in the lamina II for the lower dose, whereas in the laminae II—VI for the higher dose pretreatment. In the hippocampal sectors CA1-3 and in the hilus of the dentate gyrus even the lower dose GYKI 52466 pretreatment caused significant reduction not only in the number of the c-fos-IR cell nuclei; but also in the number of the double-labelled cells (Fig. 2). This c-fos-IR count change is even more pronounced in the granular layer of the dentate gyrus: control c-fos-IR nuclei (3535/mm^2; 100%) versus GYKI 52466-pretreatment with 25 mg/kg (1401/mm^2; 39.6% of the control) or with 50 mg/kg (184/mm^2; 5.2% of the control).

3.3. Electron microscopy

In the case of the neocortical capillary areas (Fig. 3), there was no significant difference amongst the 4-AP control and the GYKI 52466-pretreated groups. Measuring the hippocampal capillary areas (Fig. 4), there was a significant difference between the 25 mg/kg GYKI 52466-pretreated and the 4-AP control group (significant decrease in the group that received the AMPA-antagonist), whereas there was no such difference between the higher dose pretreatment and the 4-AP control group. In the neocortex (Fig. 3), the swelling of pericapillary astrocytic endfeet (i.e. the increase of the area occupied by astrocytic processes) was significantly increased in the group with lower dose of GYKI 52466-pretreatment compared to the vehicle + 4-AP controls, whereas there was no significant difference between the control and the 50 mg/kg GYKI 52466-pretreated groups. In the hippocampus (Fig. 4), no significant alteration can be detected between the three groups.

4. Discussion

The electrophysiological effects of GYKI 52466 are well-known, therefore in the present study we focused on the short-term behavioural outcomes, changes in gene expression and ultrastructural pathology, in order to asses the efficacy of the non-competitive AMPA-receptor antagonist in convulsions. The GYKI 52466 increased the latency of the first GTCS (however, without dose-dependent difference); no GTCS recurrence was observed (if there was GTCS event; only one GTCS per animal was registered during the observation). The seizure survival of the pretreated animals was 100%, however, in this case there is no significant change compared to the survival of the control group animals (80%). As for side-effects of the AMPA-antagonist pretreatment, decrease in the locomotor activity (Jakus et al., 2004) and muscle tone were observed, likely due to the formerly described central muscle relaxant effect (Tarnawa et al., 1989). Probably, this

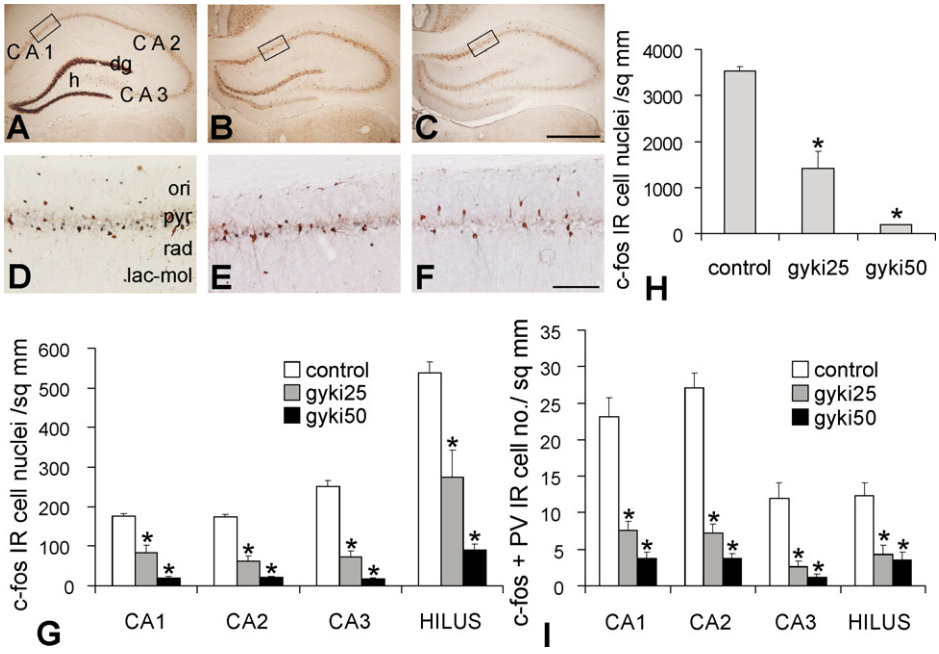

Fig. 2 Effect of GYKI 52466 on the c-fos gene expression in the hippocampus. (A—F) Representative immunohistochemical images illustrating the c-fos and c-fos + parvalbumin (PV) immunolabelled neurons in the hippocampus and dentate gyrus of the experimental animals. Animals with vehicle + 5 mg/kg 4-AP (control) (A and D); 25 mg/kg GYKI 52466 + 5 mg/kg 4-AP (gyki25) (B and E) and 50 mg/kg GYKI 52466 + 5 mg/kg 4-AP (gyki50) (C and F) treatment, respectively ($N = 4$ per group). Abbrevitations indicate the sectors of the Ammon's horn (CA1-3); the dentate gyrus (dg); the hilus of the dentate gyrus (h); and the histological layers of the hippocampus: stratum oriens (ori), stratum pyramidale (pyr), stratum radiatum (rad), stratum lacunosum-moleculare (lac-mol); respectively. Rectangular fields note the position of higher magnification samplings. Bar: 250 μm (A—C); 50 μm (D—F). Magnification: 40× (A—C); 200× (D—F). G—I. Statistical evaluation of the c-fos (G and H) and c-fos and parvalbumin (I) immunoreactive cell counts pro square millimetre (sq mm), in the pyramidal layers of the hippocampal areas and in the hilus of the dentate gyrus (G and I) and in the granular layer of the dentate gyrus (H); comparison of the data from pretreated (with 25 or 50 mg/kg GYKI 52466 before 5 mg/kg 4-AP, respectively) and the control animals (vehicle + 5 mg/kg 4-AP), 1 h after the seizure induction. Asterisks denote the significant differences (with vs. without GYKI 52466 pretreatment); (ANOVA, Bonferroni *post hoc* test, $p < 0.05$; S.E.M. is indicated in every case).

property can be responsible for the visible reduction of the tonic seizure component, and the dominance of the clonic component in the GTCS of the pretreated animals. Moreover, GYKI 52466 was found to be effective in different models of epilepsy only in doses impairing motor function (Kubová et al., 1997). However, there is a controversy about whether the GYKI 52466 lacks (Tarnawa et al., 1992; Donevan and Rogawski, 1993) or at least partially has (Block and Schwarz, 1994; Kubová et al., 1997) the effects of the ''conventional'' 1,4-benzodiazepines (suspected in the pharmacological background of the side-effects). Nevertheless, the possibility that DMSO (as vehicle, see later), eventually potentiates not only the efficacy, but also the side-effects of GYKI 52466 cannot be entirely excluded. According to literature data, higher doses are needed for the moderation of the clonic components, than for the tonic components (de Sarro et al., 1998). After the intraperitoneal administration of GYKI 52466, the peak of blood plasma level is reached between 15 and 45 min, then, with a mostly linear pharmacokinetic fashion, 100—120 min after the administration, the concentration gradually decreases to a near-zero level. Parallel with this change, the visible anticonvulsive activity of the drug is maximal between 15 and 60 min after the injection. This period can be characterised with lower seizure scores (de Sarro et al., 1998), and this is why we have focused on the first hour with maximal anticonvulsive properties of the GYKI 52466.

To explain the dose-dependent c-fos-IR differences in the neocortex; we presume (1) different activity states of the AMPA-receptors, depending on the number of modulatory molecules bound by the receptor subunits: GYKI 52466 may act on more binding sites per every subunit, in a concentration-dependent manner (Arai, 2001). In addition to, we also think that (2) the lower dose of GYKI 52466 is ineffective in the presence of the high glutamate concentration associated with the early seizure activity (Kovács et al., 2003). The GYKI 52466 seemed to be more effective in delaying the first ictal event, whereas, similarly to some studies, the propagation of the seizure activity (Barna et al., 2000) or IEG induction (Berretta et al., 1997) seemed to be facilitated in the lower dose pretreatment group. According to the literature (Hwa and Avoli, 1991; Barna et al., 2000), the AMPA-receptors are rather involved in the initiation than in the maintenance of seizure (mirrored in our behavioural data for increasing seizure latency and reducing GTCS occurrence); whereas the NMDA-receptors play crucial role especially in the maintenance and propagation of seizure (reflected in the cellular gene expression changes

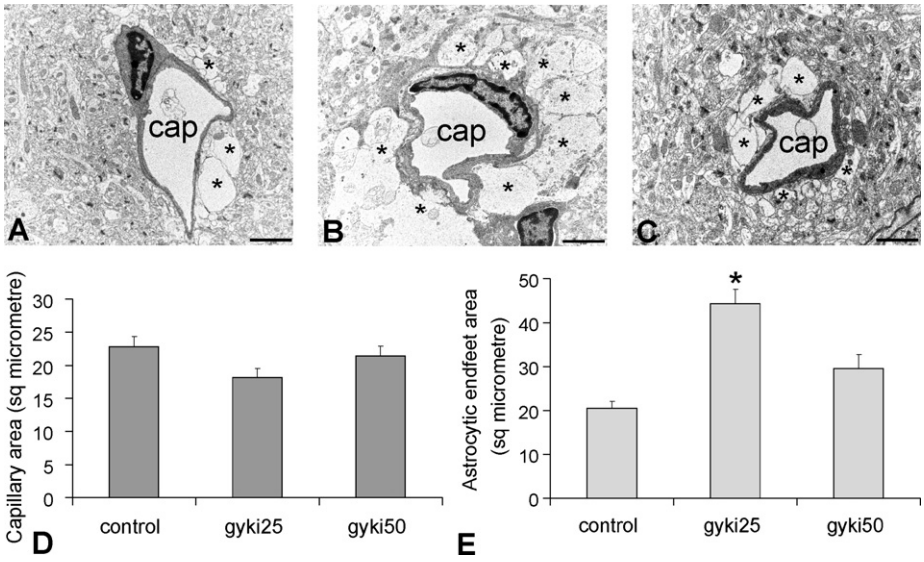

Fig. 3 Effect of GYKI 52466 on the capillary area changes and the pericapillary astrocytic process swelling in the neocortex. (A—C) Representative electron microscopic images illustrating the pericapillary astrocytic endfeet swelling in the neocortex of the experimental animals. Animals with vehicle + 5 mg/kg 4-AP (A); 25 mg/kg GYKI 52466 + 5 mg/kg 4-AP (B) and 50 mg/kg GYKI 52466 + 5 mg/kg 4-AP (C) treatment, respectively ($N = 4$ per group). Capillaries (cap); asterisks denote the surrounding oedematous astrocytic endfeet. Bar: 2 μm. Magnification: 5800×. (D) Statistical evaluation of the capillary areas in μm^2, comparison of the data from pretreated (25 or 50 mg/kg GYKI 52466) and the control animals (vehicle + 5 mg/kg 4-AP), 1 h after the seizure induction. (E) Statistical evaluation of the pericapillary astrocytic endfeet areas in μm^2, comparison of the data from pretreated (25 or 50 mg/kg GYKI 52466) and the control animals (vehicle + 5 mg/kg 4-AP), 1 h after the seizure induction. Asterisks denote the significant differences (with vs. without GYKI 52466 pretreatment); (ANOVA, Bonferroni *post hoc* test, $p < 0.05$; S.E.M. is indicated in every case).

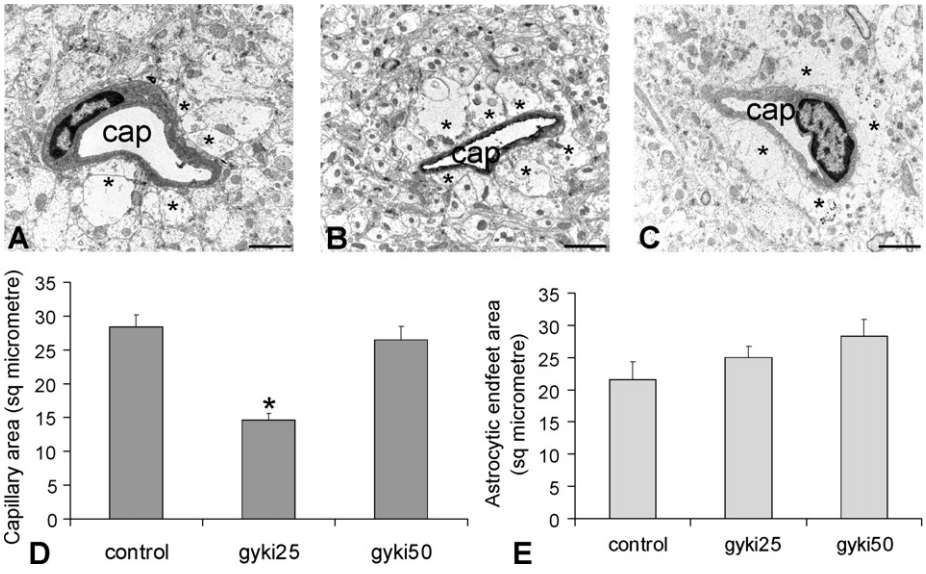

Fig. 4 Effect of GYKI 52466 on the capillary area changes and the pericapillary astrocytic process swelling in the hippocampus. (A—C) Representative electron microscopic images illustrating the pericapillary astrocytic endfeet swelling in the hippocampal areas of the experimental animals. Animals with vehicle + 5 mg/kg 4-AP (A); 25 mg/kg GYKI 52466 + 5 mg/kg 4-AP (B) and 50 mg/kg GYKI 52466 + 5 mg/kg 4-AP (C) treatment, respectively ($N = 4$ per group). Capillaries (cap); asterisks denote the surrounding oedematous astrocytic endfeet. Bar: 2 μm. Magnification: 5800×. (D) Statistical evaluation of the capillary areas in μm^2, comparison of the data from pretreated (25 or 50 mg/kg GYKI 52466) and the control animals (vehicle + 5 mg/kg 4-AP), 1 h after the seizure induction. (E) Statistical evaluation of the pericapillary astrocytic endfeet areas in μm^2, comparison of the data from pretreated (25 or 50 mg/kg GYKI 52466) and the control animals (vehicle + 5 mg/kg 4-AP), 1 h after the seizure induction. Asterisks denote the significant differences (with vs. without GYKI 52466 pretreatment); (ANOVA, Bonferroni *post hoc* test, $p < 0.05$; S.E.M. is indicated in every case).

and EM morphology alterations). The majority of the AMPA-receptors can be found in the cells of laminae II—III and V—VI (Sloper and Powell, 1978; Hicks and Conti, 1996). The efficacy of the higher dose AMPA-antagonism also refers to the importance of AMPAergic excitation in these neocortical strata. The intracortical networks are mainly mediated by NMDA-receptors, these connections remain active in spite of the presence of GYKI 52466 (Barna et al., 2000). The induction of 4-AP-induced synchronous network activity in the lower neocortical layers is rather dominated by the excitatory, whilst in the superficial ones, by the inhibitory components (Yang and Benardo, 2002). At the lower dose of GYKI 52466, therefore, we suppose (3) a local disequilibrium between the efficacy of AMPA-receptor antagonism within the inhibitory population and the overall (rather dominated by the excitatory cells) population, resulting in the overall activation status change reflected by the c-fos-IR differences. The predominance of non-NMDA receptor-mediated excitatory inputs arising from bursting neurons was shown on the fast-spiking GABAergic interneurons (Hicks and Conti, 1996); these cells are also strongly excited by thalamic afferents (Staiger et al., 1996). The inhibitory neurons — responsible for surrounding inhibition — were less activated under the influence of GYKI 52466 (significant reduction of c-fos expression at higher dose of AMPA-receptor antagonist; and showing decreasing trend at lower dose with significant reduction in lamina II). Nevertheless, we found that these interneurons possessed similar AMPA-receptor properties to those of the pyramidal cells: the 50 mg/kg dose was needed to significantly decrease the cellular activation. This local balance-shift may also be complicated by the slightly different laminar responses to 4-AP (Yang and Benardo, 2002), since the neocortical PV-containing GABAergic interneurons constitute a heterogeneous population concerning the expression pattern of voltage-gated K^+-channel subunits in the different neocortical laminae (Chow et al., 1999).

In the hippocampal areas studied, even the lower dose (25 mg/kg) of GYKI 52466 was efficient to reduce the c-fos immunoreactivity, not only in the parvalbumin-labelled, but also in the whole c-fos-IR neuronal population, especially in the granular layer of the dentate gyrus. This fact emphasises the differences in the receptorial distribution and spatial separation of excitatory and inhibitory axon systems in the hippocampus (McBain et al., 1999; Moga et al., 2002). According to immunostaining data, AMPA-receptors are concentrated in the outer molecular layer of the dentate gyrus and in the stratum lacunosum-moleculare of the regio superior (Molnár et al., 1993), while NMDA receptors are relatively scarce in the same regions (Kopniczky et al., 2005). These layers are the main excitatory input areas: the axon terminals of the perforant path synapse here (Lado et al., 2002). The prominent contribution of the AMPA-receptors to the activation of the neuronal circuits is shown by the significant reduction of the number of the c-fos-IR cells of the hippocampal CA1, CA2 and CA3 sectors and in the stratum granulosum and hilus of the dentate gyrus, even by the lower (25 mg/kg) pretreatment dose.

On the basis of the morphological results, we conclude that acute seizures caused the increased swelling of the astrocytes. Our previous investigations (Fabene et al., 2006) revealed the importance of brain swelling in 4-AP seizures: the long-lasting astrocyte swelling was responsible for the critical decrease of apparent diffusion coefficient (ADC) values, as shown by our MRI experiments. Regional differences in astrocytic swelling (Fabene et al., 2006) are likely due to the various astrocytic capacities for glutamate metabolism, neurotransmission (Bordey and Sontheimer, 2000) and aquaporin-synthesis (Han et al., 2004). The swelling of the astrocytes (Kimelberg, 2004) obliterates brain extracellular spaces, inhibiting the clearance of transmitters, ions and HCO^{3-} from the extracellular space; contributing to and enhancing the cellular damage (van Gelder et al., 1983); leading to a long-lasting decrease in volume transmission (Syková, 2005; Fabene et al., 2006). The current antiepileptic drugs have been shown to exert their effect on the astrocytes by directly depressing their Ca^{2+}-signalling (Tian et al., 2005). The mechanism of seizure-related astrocyte swelling is likely to be multifactorial (Olsson et al., 2006); such as (1) circulatory (Hong et al., 2004) and (2) metabolic (Briellmann et al., 2005) changes due to seizure activity. Alternatively, the astroglial volume-change may be secondary to (3) the failure of these cells to manage the consequences of increased neuronal activity (e.g. may also derive from fluid uptake secondary to the extracellular changes accompanying elevated neuronal functioning), thus providing a mechanism reinforcing seizure *per se* (Olsson et al., 2006). Literature data are limited concerning the roles of AMPA-receptor antagonism in the seizure-related pericapillary astrocyte swelling; whilst the action of the NMDA-receptor antagonists influencing the changes of the brain water compartments (Hantzschel and Andreas, 1998), or modulating the pericapillary astrocytic swelling following injuries (Trout et al., 1995) or glutamate-administration (Bender et al., 1998) have been already described. In our present experiments, the AMPA blockade was completely ineffective in decreasing seizure-related astrocyte swelling—in the neocortex GYKI 52466 increased the swelling of the glia. Other studies (Lees and Leong, 2001) also question the protective effect of GYKI 52466 against seizure-related morphological damage, which is a contrasting feature with the anticonvulsant efficacy of this compound (Borowicz et al., 2001; Gulyás-Kovács et al., 2002). On the other hand, recent results from our laboratory proved that in the same experimental conditions NMDA blockade with MK801 decreased brain oedema significantly (Zádor and Weiczner, unpublished), indicating the differences between roles of the ionotropic receptors, and the significance of glutamate. Thus, the overactivation of NMDA-receptors is suggested mainly in the pathophysiological background of the morphological changes of the 4-AP paradigm (Peña and Tapia, 1999). On the basis of these results, we conclude that astrocyte swelling is not mediated by AMPA-receptors, and blockade of the AMPA-receptor does not protect against astroglial swelling in epilepsy. Further investigation should elucidate the role of astrocytic glutamate receptors, transporters and other cellular factors (such as aquaporins and connexins) in the 4-AP-elicited acute convulsions and the pathophysiology of astrocytic oedema associated with generalised tonic-clonic seizures.

The effects of DMSO on glutamate responses (Lu and Mattson, 2001; Tsvyetlynska et al., 2005) should be taken into account in interpreting the results of experiments in

which DMSO is used as a solvent (Santos et al., 2003). DMSO, as a drug vehicle increases the drug concentration into the extracellular space, but since the BBB permeability is increased, it may also provide an avenue for development of vasogenic oedema (Kleindienst et al., 2006). Taken together, the oedematous changes in the pericapillary territories may partially due to the side effects of the vehicle used in this experiment. This point can be hardly clarified without using other vehicles. Other methods for dissolving GYKI 52466, such as using distilled water (Block et al., 1996), physiological saline (Kubová et al., 1997), or acidic solutions titrated up to neutral pH (Barna et al., 2000) were proven equally irreproducible in our laboratory. According to the literature (Lees and Leong, 2001) and to our experience, as well, GYKI 52466 was insoluble in water, and instable in solutions above pH 4. In our next fMRI and EM experiments 2-hydroxypropyl-β-cyclodextrin (HPCD) (Jakus et al., 2004) or CREMOFOR EL (Berretta et al., 1997) as a much more inert vehicle than DMSO is planned to be used. Nevertheless, in another GYKI 52466 study, the HPCD elevated the AMPA/kainate toxicity by increasing the amount of seizure damage (Lees and Leong, 2001).

Summarizing our results, it seems that the main protective effect of GYKI 52466 is based on the moderate inhibition of seizure activity only; although the relatively short duration of action may also have contributed to the limited effects of GYKI 52466. This notion-concerning the low therapeutic index of GYKI 52466- is supported by the literature (Lees and Leong, 2001; Jakus et al., 2004). The different extent of participation of AMPA-receptors in organising neuronal circuits of neocortex and hippocampus in convulsions is reflected by the dissimilar GYKI 52466 efficacy in reducing the seizure-related neuronal activation. In the hippocampus, cellular activation was relatively more dependent upon AMPA-receptors than in the neocortex, but astrocyte swelling was not. We think therefore, in accordance with the literature (Berretta et al., 1997; Barna et al., 2000; Jakus et al., 2004) that the AMPA-receptors appear to be involved primarily in the intiation of seizures compared with the maintenance and propagation of cortical seizure activity. Based on the present as well as on previous findings (Peña and Tapia, 1999; Lado et al., 2002; Zádor and Weiczner, unpublished), antagonists of NMDA receptors play a more prominent role than antagonists of AMPA-receptors in the 4-AP model in preventing or reducing acute morphological changes such as astrocytic swelling.

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