IONOTROPIC GLUTAMATE ANTAGONISM IN THE 4-AMINOPYRIDINE RAT CONVULSION MODEL: THE MORPHOLOGICAL AND FUNCTIONAL ASPECTS OF THE ACUTE SEIZURE

Summary of Ph. D. Thesis

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LIST OF ABBREVIATIONS

4-AP	4-aminopyridine
ADC	apparent diffusion coefficient
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazol propionate
ANOVA	analysis of variance
AOI	area of interest
AQP4	aquaporin-4
BBB	blood-brain-barrier
CA	cornu Ammonis
DMSO	dimethyl-sulphoxide
DWI	diffusion-weighted imaging
EEG	electroencephalography
EM	electron microscopy
GABA	γ-amino-butyric acid
GLU	glutamate
GTCS	generalised tonic-clonic seizure
i.p.	intraperitoneal
IEG	immediate early gene
IR	immunoreactive
fMRI	functional magnetic resonance imaging
NMDA	N-methyl-D-aspartate
PCR	polymerase chain reaction
PV	parvalbumin
RARE	rapid acquisition-rapid enhancement
rCBF	regional cerebral blood flow
rCBV	regional cerebral blood volume
ROI	region of interest
SE	status epilepticus
T2	transverse relaxation time
T2W	T2-weighted (imaging)
TE	echo time
TR	repetition time
vs.	versus

1. INTRODUCTION

The excessive, pathologic oversynchronisation of neuronal activity and the disequilibrium between certain excitatory and inhibitory factors 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 whereas the inhibition of the excitatory responses is under current research, but results are mainly available about the role of NMDA -mediated events.

One of the well-known 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 and pharmacodynamic properties. 4-AP is a nitrogen-containing heterocyclic K^+-channel blocker, exerting its effect mainly via IK(A) and IK(V) channel types, 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, the latter are opened by the mainly presynaptically acting 4-AP-induced membrane-depolarisation. The sustained, repeated, synchronised neuronal discharge, the so-called “burst firing” is a basic feature of the central nervous system seizure mechanism. The prolonged neuronal depolarisation stimulates both excitatory and inhibitory neurotransmitter release, especially glutamate (GLU) and reinforces the inhibitory and excitatory postsynaptic potentials.

According to our microdialysis investigations, fast and highly significant elevation can be demonstrated in the striatal concentration of GLU. Since many of the excitatory afferents of the striatum originate from the cerebral cortex (and thalamus), we may suppose that the elevation in the striatal GLU is consequence of neocortical hyperactivity and the excessive release of the transmitter from corticostriatal (and thalamostriatal) axon terminals. Interestingly, the GLU level reaches its maximum at 120 min, well after the cessation of the electrographic seizure. This explains the long-lasting spiking activity on the striatal EEG and raises the possibility that besides the postsynaptic depolarization, GLU might regulate the release of other transmitters through metabotropic GLU receptors.

The immediate early gene (IEG) *c-fos* is an inducible transcription factor playing important role in certain nuclear regulative processes. The gene products of the IEGs, via transcriptional regulation influence the genes involved in the maturation and adaptation mechanisms of the nervous system, or in plasticity and neurodegeneration, as well as in epilepsy. The full spectrum of the genes regulated by *c-fos* is not known as a whole. Nevertheless, the signal transduction characteristics and the intracellular pathways of *c-fos* gene expression are basically understood. The *c-fos* mRNA transcription shows strict correlation with the electrophysiologically proven cellular activity, 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. NMDA and AMPA receptors are amongst the external signals, which activate *c-fos* transcription.

Amongst the earliest neuropathological changes in seizure activity, the swelling of the astrocytic processes has already been observed. Astrocytes are the main cell types that swell in cytotoxic brain oedema, especially the pericapillary foot processes, which are the predominant sites of aquaporin-4 (AQP4) expression in the brain. Glutamate, at similar concentrations required to induce neuronal cell death, has been shown to increase cell volume in cultured astrocytes. The astrocytic swelling has numerous deleterious secondary effects, such as the release and decreased uptake of excitatory amino acids; worsening the micro-environmental circumstances like a *circulus vitiosus* process. Pharmacological inhibition of seizure activity decreases brain oedema in different animal models. Beside the electron microscopic investigations, the importance of the *in vivo* imaging techniques, such as functional magnetic resonance imaging is continuously increasing.

Studies involving intracellular recording in rat neocortical slices have shown that NMDA receptors contribute to the process of stimulus-induced paroxysmal depolarisation shift (PDS) amplification by prolonging the duration and reducing the latency of the epileptiform discharge. The high-affinity open-channel NMDA receptor blocker dizocilpine maleate (MK-801) is also potent anticonvulsant and protects against seizure-related brain damage. The MK-801 is widely used for NMDA receptor mapping in imaging studies; or to establish an experimental schizophrenia in animal models.

The compound GYKI 52466 is a selective, non-competitive, presumably allosteric antagonist of the AMPA-subtype ionotropic glutamate receptors; with good blood-brain-barrier (BBB) permeability, neuroprotective effects in seizure and cerebral ischaemia, antinociceptive and anti-inflammatory properties and has other advantageous effects in ischaemic conditions. Electrophysiological investigations proved the antagonistic activity of GYKI 52466 upon the AMPA and kainite receptors, whereas practically no effect has been demonstrated on the NMDA receptors, metabotropic glutamate receptors, and on the $GABA_A$-receptors.

Synapses in cerebral cortex and hippocampus commonly coexpress NMDA and AMPA receptors. According to this observation, the proconvulsive feature of glutamate-receptor agonists; and the anticonvulsive effect of the glutamate-receptor antagonists have been proved in several *in vivo* and *in vitro* models of experimental epilepsy.

In our former studies 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 latency of the GTCS was significantly increased (dextrometorphan, ketamine) or the GTCS occurrence was significantly reduced (MK-801), as well.

2. OBJECTIVES

A. Based on the success of our cooperating partners in the fMRI description of the pilocarpin epilepsy paradigm, we proposed a pilot experiment for the fMRI study of the 4-AP acute convulsion model concerning the changes in the regional cerebral blood flow (rCBF) and apparent diffusion coefficient (ADC) regarding the water movements (extra- and intracellular oedema).

B. The aim of the AMPA receptor antagonism study was to assess the antagonism of another well-characterised glutamate receptor, the AMPA receptor in a similar (4-AP-induced acute convulsion) paradigm. According to the time-course of the GYKI 52466 anticonvulsive efficacy, 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 representing a subpopulation of GABAergic interneurons.

C. The NMDA antagonists significantly decreased seizure-related astrocyte swelling in the cerebral cortex, so we are going to compare the efficacy of NMDA and AMPA receptor antagonism on the astrocyte swelling and neuronal activation (c-fos expression) associated with acute convulsions.

3. MATERIALS AND METHODS

3.1 Functional and structural MRI measurements in the 4-AP paradigm

The MRI experiments were performed in the Experimental MRI Laboratory of the Department of Anatomy and Histology, Faculty of Medicine, University of Verona, Verona, Italy. Male adult Wistar rats (80–90 days of age) were kept under controlled environmental parameters and veterinarian control. The experiments received authorization from the Italian Ministry of Health. Rats were randomly divided into two groups: in twelve rats, seizures were elicited with a single intraperitoneal (i.p.) bolus of 4-AP (5 mg/kg), dissolved in physiological saline (0.67 mg/ml). Control animals (n = 12) received the same volume of physiological saline i.p. MRI analysis was performed 2 h, 24 h and 3 days after seizure arrest. Twelve animals for both conditions were analysed with structural (T2W and DWI) and functional (rCBV) MRI. For

MRI data, difference between DWI, T2W and rCBV values obtained in control vs. 4-AP-treated rats was evaluated with one-way analysis of variance (ANOVA) for repeated measures followed by the LSD post-hoc test, setting the significance at $p < 0.05$.

3.2 Administration of the glutamate-receptor antagonists

The animal experiments were conducted in accordance with prevailing laws and ethical considerations. 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-AP was administered i.p., in 5 mg/kg dose. The non-competitive NMDA receptor antagonist MK-801 was dissolved in physiological saline and administered i.p. in a volume of 1 ml, 10 min prior to the application of 4-AP in 1 mg/kg dose. The non-competitive AMPA receptor antagonist GYKI 52466 was dissolved in 50% DMSO (dimethyl-sulphoxide) dilution (3.33 or 6.67 mg in 1 ml vehicle), and administered i.p. 15 min before 4-AP injection, in 25 or 50mg/kg dose, respectively.

For the MK-801-pretreatments, the animals were randomly divided into three groups. In the first group, the animals (n=18) were pretreated with 1 mg/kg MK-801; and 10 min later, the convulsant 5 mg/kg 4-AP was administered. The control group (n=18) received the solvent of MK-801 and 5 mg/kg 4-AP. The experiments were finished 1, 3 and 24 h after the 4-AP injection (6-6-6 animals pro every time group). At the end of the experiments, three-three animals from every group were sampled for PCR or were processed for electron microscopy, respectively.

For the GYKI 52466-pretreatments, the animals were randomly divided into three groups. In the first two groups, the animals (10 per group) were pretreated with 25 mg/kg 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.

For the immunohistochemical evaluation of the possible individual c-fos expression effect of the applied antagonists, another four control groups have been established (N=4 in each group), as follows: animals receiving physiological saline only (1 ml for an animal with 200 g bodyweight), animals receiving 50 mg/kg GYKI 52466 only, animals receiving 1 mg/kg MK-801 only and animals receiving 5 mg/kg 4-AP only. These animals have been sacrificed 1 h after the injection and processed for immunohistochemistry or electron microscopy as described below.

The behavioural outcome of the pretreatment with the AMPA antagonist was observed up to 1h after the 4-AP injection. The onset of the GTCS was always sudden and clear-cut, so the

latency 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).

On the coronal brain slices *c-fos* and parvalbumin double-labelling immunohistochemistry was carried out. Quantitative analysis was performed on five sections per animal (N=4; the mean data per each animal was used in the statistical analysis). 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. 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.

Samples of the right parietal neocortex *(for MK-801 experiments)*; or right parietal neocortex and hippocampus *(for GYKI 52466 experiments)* were prepared for electron microscopy. These 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). Approximately 900 μm^2 of sample surface was viewed systematically through all neocortical layers of the parietal cortex or in the hippocampus; 4 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. 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).

4. RESULTS

4.1 Structural MRI of the 4-AP model

4.1.1 T2 maps

Quantitative T2 maps calculated from multi-echo spin-echo acquisitions clearly showed the overall pattern of changes in control and epileptic rats (both at 2 h and 24 h), highlighting the signal increase throughout the cerebral cortex. The T2 value was also slightly increased in the amygdala. In the diencephalon, selective increase in the T2 signal was documented in medial thalamic regions. T2 values exhibited a 56% increase ($p<0.001$) in the parietal cortex at 2 h vs. control; this increase was still present ($p<0.01$) at 24 h (+29%). A dramatic increase

in the T2 values was found also in the hippocampus after 2 h (+72%, $p<0.001$) and after 24 h (25%; $p<0.01$). The temporal cortex showed the same pattern of alteration (+97%; $p<0.0001$ at 2 h; + 39%, $p<0.001$ at 24 h). A highly significant ($p<0.001$) increase in T2 (52% at 2 h and 33% at 24 h) was also found in the medial thalamus. In all of these structures, the T2 values observed 24 h after seizures were significantly higher than those observed in the controls ($p<0.001$). No evident differences were detected 3 days after seizure induction.

4.1.2 Diffusion-weighted imaging

Diffusion-weighted images, based on the sensitization of the MRI signal to the Brownian motion of water molecules, reveal maps of brain tissue water motion, resulting in hyperintense alterations. ADC maps of brain tissue water, calculated from DWI, showed consistent changes in ADC in the cerebral cortex (parietal cortex), hippocampus and amygdala of the epileptic animals at 2 h and 24 h compared with control ones. An evident reduction of the average ADC value was documented in the parietal (-44%) and temporal (-34%) cortices, hippocampus (-23%) and medial thalamus (-11%) 2 h after 4-AP-induced seizures. A further drop of ADC value was documented 24 h after 4-AP injections in the hippocampus (-46%), medial thalamus (-21%) and in the cerebral cortex (parietal: -57%; temporal: -64%). ADC values returned to basal levels 3 days after seizure onset.

4.2 Functional MRI of the 4-AP model: *rCBV maps*

Quantitative parametric maps reconstructed by images acquired with gradient-echo sequence before and after USPIO administration in control and epileptic animals at 2 h and 24 h showed an increase of average blood volume value in the early stages of the seizures, with a progressive normalization of the values at 24 h in the parietal cortex and hippocampus. In the medial thalamus, the value failed to return to baseline 24 h post-convulsions ($p<0.001$). 2 h after seizure onset, the values were higher in the parietal cortex (+32%, $p<0.0001$) and in the hippocampus (+16%, $p<0.01$) versus control. At subcortical levels, rCBV increased in the medial thalamus (+44%, $p<0.0001$). 24 h after seizures, rCBV showed marginal alteration in the parietal cortex (+4%) and hippocampus (+4%) but was still highly significantly ($p<0.0001$) increased (+21%) in the medial thalamus. No significant alterations were found 3 days after seizure onset.

4.3 Behavioural analysis of the pretreatments with GYKI 52466

The i.p. administration of 4-AP causes characteristic behavioural symptoms within 15-20 min: 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. The increasing motor symptoms lead to a “wild running” phenomenon and, finally, to generalised tonic-clonic seizures (GTCS), often preceded by vocalisation. With the dissipation of the tonic component, the clonic one becomes more dominant for a while with tenebrosity for other 10-15 min until regaining normal activity. The gradual dissipation of the seizure activity can be detected electrophysiologically after 50-60 min; 90-100 min after the 4-AP injection, the animals recover. Nevertheless, approximately 20% percent of the control group animals die during or after GTCS, due to the seizure.

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. 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-hour 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.

4.4 Immunohistochemistry of the pretreatments with GYKI 52466

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. 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 in the lamina V only, 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 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. This *c-fos*-IR count change is even more pronounced in the granular layer of the dentate gyrus: control *c-fos*-IR nuclei (3535 per mm^2; 100%) vs. GYKI 52466-pretreatment with 25 mg/kg (1401 per mm^2; 39.6% of the control) or with 50 mg/kg (184 per mm^2; 5.2% of the control).

The control groups (receiving physiological saline only or 50 mg/kg GYKI 52466 only or 1 mg/kg MK-801 only; without seizure induction with 4-AP) show no significant difference compared to one another, so the effect of antagonists given alone does not differ statistically from that of the physiological saline in this acute convulsion paradigm.

4.5 Electron microscopy of the pretreatments with MK-801 or GYKI 52466

The swelling of pericapillary astrocytic endfeet (i.e. the increase of the area occupied by astrocytic glia limitans) was significantly reduced in the groups with *MK-801-pretreatment* compared to the 4-AP controls at any observed time point. Regarding the capillary areas, there was no significant difference between the animal groups with and without MK- 801-pretreatment at 1h and after 24h. The only significant change at 3h indicates that the MK-801-pretreatment significantly reduced the capillary lumen compression due to pericapillary oedema. This difference is also significant between the 1h and 3h pretreated groups.

In the case of the *neocortical capillary areas*, there was no significant difference amongst the 4-AP control and the *GYKI 52466-pretreated* groups. The swelling of pericapillary astrocytic endfeet in the neocortex 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.

Measuring the *hippocampal capillary areas*, 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 hippocampus, no significant alteration can be detected between the three groups.

5. DISCUSSION

5.1 Functional and structural MRI in the 4-AP acute convulsion paradigm

Structural alterations were studied by T2W RARE images that were already reported to be sensitive in detecting epileptic alterations. T2W maps indicate that the peak of T2 alterations occurs 2 h after seizures, whereas 24 h after seizures, these values are decreased close to baseline levels. In the chronic epilepsy models, the alterations of the T2W maps are more evident at 24 h after seizures, compared to our present findings in the 4-AP model. This fast recovery in T2W images would be consistent with the absence of an epileptogenic phase and reflected a reduced severity of the oedema following brief, acute seizures. The ADC alterations reflect pathological conditions in brain tissue that are only partially understood and involve changes in the diffusion characteristics of intra- and extracellular water compartments, water exchange across permeable boundaries and changes in volume transmission. In general, reduction of the ADC has been associated with acute cytotoxic oedema. The present findings of decreased ADC values in the cerebral cortex can be explained with cytotoxic oedema. The swelling of the astrocytes, and the oedema of the perivascular glia limitans, suggest the presence of excess amounts of excitatory transmitters, metabolites (CO_2) and K^+ in the extracellular space. On the basis of the morphological results, we conclude that the brief, acute seizures caused cellular oedema of the astrocytes mainly. The swelling of the astrocyte obliterated brain extracellular spaces; inhibiting the clearance of transmitters, ions and HCO_3^- from the extracellular space, contributing to and enhancing the cellular damage. However, the astrocyte that has taken up glutamate from the extracellular space may release it again through connexon-hemichannels. Therefore, the astrocyte may sustain a long-lasting decrease in volume transmission. These changes may explain the long-lasting decrease of the ADC values in our experiments. The decrease of the capillary diameter impairs the local microcirculation and could contribute to the decrease of ADC values. The partial mismatch between the altered DWI values and the minor astrocytic swelling detected by histological analysis at 24 h may be partially explained by a dehydration process that affects the oedema evaluation, allowing only the detection of the more obvious alterations. The increase of rCBV at the same time may reflect the compensatory effects of the local changes at the level of the arterioles and larger vessels. In order to better understand CBV data, it should be considered that oedema results in the decrease of the diameter of microvessels in the affected regions, thus inducing a relative ischaemia, and a compensatory hyperperfusion in adjacent areas may be hypothesized. Taken together, our data show that brain damage following SE involves several presumably pathological processes operant in both limbic and extra-limbic regions, suggesting that sustained seizure

activity elicits a complex rearrangement of cortical and subcortical neural networks. The ultrastructural changes indicate different processes controlling diffusion properties of the extracellular spaces.

5.2 Effect of the pretreatment with glutamate-receptor antagonists on the seizure-associated symptoms and seizure outcome

According to our former results, the non-competitive NMDA receptor antagonists MK-801 (given in pretreatment) significantly reduced the number of animals displaying GTCS without prolonging the seizure-latency. After the intraperitoneal administration of MK-801 the first characteristic symptoms: muscle hypotonia and unsteady gait, slight impairment of postural control with stereotypic, repeated movements of the head; due to the psychotomimetic (phencyclidine-like) side effects of MK- 801 developed, followed by mild tremor. The animals treated with MK-801 rarely developed generalized tonic-clonic convulsions; muscle tremor being the main symptom throughout the experiment.
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 and muscle tone were observed, likely due to the formerly described central muscle relaxant effect. Probably, this 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. Nevertheless, the possibility that DMSO 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.

5.3 Effect of the pretreatment with glutamate-receptor antagonists on the seizure-associated neuronal activation

MK-801 caused an overall decrease of Fos immunoreactivity (layers II-III, IV, VI, less markedly in layer V). Pretreatment with MK-801 resulted in a significantly lower number of Fos-labelled neurons in CA1, CA2 and CA3 regions of the Ammon's horn with respect to the animals that had received 4-AP only. Pretreatment with MK-801 reduced Fos

immunoreactivity in the dentate granule cell layer, as confirmed by statistical evaluation. Moreover, MK-801 resulted in a significant decrease of the number of Fos-containing cell nuclei in the dentate hilus. When given alone, the MK-801 caused only minimal cortical and hippocampal Fos induction. The number of stained cells induced by MK-801 was consistently very low, without significant differences.

As for 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. 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. The GYKI 52466 seemed to be more effective in delaying the first ictal event, whereas, the propagation of the seizure activity or IEG induction seemed to be facilitated in the lower dose pretreatment group. According to the literature, 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 and EM morphology alterations). The majority of the AMPA receptors can be found in the cells of laminae II-III and V-VI. 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. 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. 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; these cells are also strongly excited by thalamic afferents. The inhibitory neurons responsible for surrounding inhibition were less activated under the influence of GYKI 52466. 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, 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.

In the hippocampal areas studied, even the lower dose 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. 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, while NMDA receptors are relatively scarce in the same regions. These layers are the main excitatory input areas: the axon terminals of the perforant path synapse here. 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.

5.4 Effect of the pretreatment with glutamate-receptor antagonists on the seizure-associated pericapillary astrocytic swelling

Our investigations revealed the importance of brain swelling in 4-AP seizures: the long-lasting astrocyte swelling was responsible for the critical decrease of ADC values, as shown by our MRI experiments. Regional differences in astrocytic swelling are likely due to the various astrocytic capacities for glutamate metabolism, neurotransmission and aquaporin-synthesis. The mechanism of seizure-related astrocyte swelling is likely to be multifactorial; such as **(1)** circulatory and **(2)** metabolic 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, thus providing a mechanism reinforcing seizure *per se*. 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 glia-to-neuron signalisation processes or the changes of the brain water compartments, or modulating the pericapillary astrocytic swelling following injuries or following glutamate-administration have already been described. The glutamate-induced astrocytic swelling can be moderated with MK-801. In accordance with these data, we demonstrated the long-term astrocytic oedema-reducing effect of the MK-801-pretreatment in the 4-AP-induced seizure. The capillary area enlargement was limited to the 3h pretreatment group; this alteration may reflect a short-term perfusion change at the affected sites.
In our GYKI 52466 experiments, the AMPA blockade was completely ineffective to decrease the seizure-related astrocyte swelling – in the neocortex GYKI 52466 even increased the swelling of the astroglia. Other studies 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. On the other hand, the above described results from our laboratory proved that in the same experimental conditions NMDA blockade with MK-801 decreased brain oedema significantly, 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 background of the morphological changes of the 4-AP paradigm.

6. CONCLUSIONS

In our pilot study we have described the properties of the 4-AP-evoked seizure activity in the rat brain by means of structural and functional MRI. These are the ***first MRI data about the 4-AP acute convulsive model***, providing information for further experiments about the possible treatment options in this rat convulsion paradigm.

On the basis of these results, we conclude that astrocyte swelling is unlikely to be 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 in the 4-AP-elicited acute convulsions and the pathophysiology of astrocytic oedema associated with generalised tonic-clonic seizures.

Summarizing our results, it seems that ***the main protective effect of GYKI 52466 is based on the moderate inhibition of seizure activity only*** (increasing the latency of the GTCS, and decreasing the lethality in the animal groups); 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. Our data suggest that ***the seizure-associated cellular damages (such as astrocytic swelling) depend critically rather on the participation of NMDA receptors***, than on the AMPA receptors. ***In the hippocampus, cellular activation was rather dependent on AMPA receptors than in the neocortex***, but astrocyte swelling was not. We think therefore, in accordance with the literature that the AMPA receptors are rather involved in the initiation, than in the maintenance and propagation of cortical seizure activity. 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. By our experience in the 4-AP convulsion paradigm, rather the antagonists of the NMDA than AMPA receptors play crucial role in circumventing the acute morphological changes, such as astrocytic swelling.

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LIST OF *IN EXTENSO* PUBLICATIONS RELATED TO THE THESIS

I. Szakács, R., **Weiczner, R.**, Mihály, A., Krisztin-Péva, B., Zádor, Zs., Zádor, E.: Non-competitive NMDA receptor antagonists moderate seizure-induced *c-fos* expression in the rat cerebral cortex. *Brain Res Bull 59: 485-493, 2003* ***IF: 1.943***

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