SUMMARY OF PH.D. THESIS

THE EFFECTS OF GLOBAL BRAIN ISCHEMIA ON THE CORTICAL ACTIVITY OF TWO DIFFERENT RAT STRAINS AND INVESTIGATION OF A NEW POSSIBLE NEUROPROTECTIVE STRATEGY

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List of abbreviations

- 4VO:4-vessel occlusion
- BSR: burst suppression ratio
- CA1: cornum ammon region 1
- CBF: cerebral blood flow
- CCA: common carotid artery
- DHEA: dehydroepiandrosterone
- EAAT: excitatory aminoacid transporter
- ECoG: electrocorticogram
- fEPSP: field-excitatory postsynaptic potential
- FJ-C: Fluoro Jade C
- FB: first burst
- Glu: glutamate
- GOT: glutamate-oxaloacetate transaminase
- I/O: input/output
- ISF: interstitial fluid
- LTP: long-term potentiation
- NF-κB: („nuclear factor kappa-light-chain-enhancer of activated B cells")
- NMDA: N-methyl-D-aspartate
- OxAc: oxaloacetate
- SD: Sprague-Dawley
- TBS: theta burst stimulation
- TNFα: tumor necrosis factor-α

Introduction

The annual number of deaths worldwide is approximately 60 million, 60% of which are caused by noninfectious diseases such as cancer or neurodegeneration. Half of these cases involve malfunctions of the cardiovascular system. Disturbance of the blood supply of the brain is one of the most frequent cerebrovascular diseases. Only a particular area of the brain is involved during hemorrhagic and ischemic stroke, but the whole body, including all regions of the brain, suffers from the lack of oxygen and substrates when cardiac arrest occurs. Accordingly, different experimental models of ischemia are required which correspond to the various clinical pathophysiological situations. The 4-vessel-occlusion (4VO) model is suitable for inducing global cerebral ischemia ischemia in experimental animals. Many independent research groups have reported significant discrepancies between the data recorded in human pathophysiological states and the data obtained from animal experiments. It should be borne in mind that there is a considerable diversity in cerebral vascular architecture in the different species, and there are also interstrain and even intrastrain anatomical differences in the vasculature of rats. There are striking differences in the physiological functions of Sprague-Dawley (SD) and Wistar rat strains at many levels, e.g. behavior, learning and memory, enzymatic activity, gene expression and focal ischemic injury. Both Wistar and SD rats are widely used in experiments involving global ischemia, such as 4VO. No previous data demonstrate whether any difference in cerebral cortical activity and the consequent histological changes can be observed following global cerebral ischemia in Wistar and SD rats supplied by the same vendor (Charles-River Laboratories). It should also be emphasized that such differences can greatly influence the results of experiments and even the clinical application of neuroprotective strategies.

We discovered striking differences between SD and Wistar rats when we applied 4VO surgical intervention. It has already been published that a highly increased cytosolic concentration of Ca^{2+} ions plays a prominent role in the functional and cellular loss after ischemia. A second elevation of Ca^{2+} occurs after the ischemic insult, which is responsible for the apoptotic effects following hypoperfusion. Free radicals are formed as a result of the

reduced blood flow. These influence the excitatory transmitter release of neurons which can enhance the processes of free radical formation. Thus, if there are huge discrepancies in the cerebral blood flow (CBF) of SD and Wistar rats during 4VO, this can explain the differences seen between the two rat strains.

In a second series of experiments we investigated a possible new neuroprotective strategy applied as combined treatment with two endogenous compounds. The treatment was designed to moderate both the early and the late ischemic events and to protect the hippocampal pyramids from the loss of synaptic function and from cell death. The excess glutamate (Glu) which appears in the interstitial fluid (ISF) immediately after the start of ischemic period, increases the intracellular Ca^{2+} concentration through the activation of N-methyl-D-aspartate (NMDA) receptors. The high Ca^{2+} concentration leads to the release of Ca^{2+} from the intracellular storages. Although glial cells have their excitatory amino acid transporters (EAAT) activated, these work in a reversed way when the Glu level is extremely high. The membrane potential of the endothelial cells with EAAT transporters in their membrane is much more stable during ischemic conditions and may therefore reduce the excess Glu when ischemia occurs. It is reasonable to say that the extremely high Glu level of the ISF could be a valuable point of attack a neuroprotective strategy. The concentration gradient between the ISF and the blood plasma can be increased by decreasing the Glu concentration of the blood plasma by activating the glutamate-oxaloacetate transaminase (GOT) enzyme. Through its transaminase activity GOT converts glutamate to 2-α-ketoglutarate and oxaloacetate (OxAc) to aspartate. The Glu efflux from the brain to the blood is enhanced when the Glu level in the blood plasma decreases. This is the phenomenon of Glu scavenging. Early consequences of the ischemic insult are handled by the Glu-scavenger OxAc, but late ischemic events such as inflammatory events take place a few hours or days after the insult. During these processes the affected area is characterized by elevated interleukin-1 and interleukin-6 concentration. The expression of tumor necrosis factor α (TNFα)-activated NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which regulates the major genes responsible for inflammation, cell proliferation and apoptosis, is also increased. Dehydroepiandrosterone (DHEA), an endogenous neurosteroid, has been proved to moderate inflammation due to its numerous neuroprotective effects. DHEA is capable of decreasing the activation of NF-κB and the level of interleukins. Under ischemic conditions, when the extremely high intracellular Ca^{2+} concentration is close to its cytotoxic level, DHEA treatment can prevent Ca^{2+} ions from flowing into the mitochondrial matrix. Furthermore, DHEA activates Akt kinase, a serine-threonine kinase, which is known to contribute to inhibition of the apoptotic cascade in its active form.

With our combined treatment, we are able to moderate both the early ischemic and the late ischemic events following an ischemic period which can result in significant functional and cell loss. Thus, the application of combined treatment to diminish excitotoxic and inflammatory processes is expected to lead to much more effective neuroprotection.

Aims

1. We discovered marked interstrain differences between rats from the Wistar and from the SD strains when we performed 4VO global ischemia surgery. It is well known that there are striking differences in the physiological functions of the two rat strains at many levels, e. g. behaviour, learning and memory, enzymatic activity, gene expression. We hypothesize that there is a huge difference in the vascular anatomy of the two strains. Therefore we expect the 10 min of global cerebral ischemia to result in other types of cortical activity and in different magnitude of cell loss**. In order to clarify the origin of these discrepancies we set to carry out *in vivo* electrophysiological (ECoG) and histological (Fluoro Jade C, Evans Blue and Cresyl violet) experiments.**

2. We are planning to examinate the neuroprotective effects of OxAc on 4VO Wistar rats. Transient global cerebral ischemia with a duration of 10 min is going to be performed and we investigate the neuroprotection of Glu scavenging in comparison to the reduced synaptic plasticity after 4VO. OxAc treatment is capable of moderating the impairment of synaptic plasticity after ischemia according to our previous results. In order to decrease the dose of OxAc, we administer DHEA as another endogenous compound. Thus we would apply a combined treatment in order to decrease the damage caused by both the early and the late ischemic events after 4VO. **We investigated the neuroprotective effects of OxAc in *in vivo* electrophysiological experiments (ECoG recordings), and the effectiveness of the combined treatment in *in vitro* electrophysiological experiments (LTP measurements).**

Materials and methods

Animals and housing conditions

Male Charles River rats of the Wistar strain (N=80) and of the Sprague-Dawley strain (N=20) were used. Animals weighing 200–300 g were applied. They were maintained on food and water, *ad libitum*. Every effort was made to minimize animal suffering and the number of sacrificed animals. The protocols for animal care approved by both the Hungarian Health Committee and the European Communities Council Directive of 24 November 1986 (86/609/EEC) were followed.

The 4VO ischemic modell

Preparation of the transient global cerebral ischemia model (4VO)
The 4VO procedure was performed under sodium pentobarbital anesthesia (60 mg/kg, i.p.). Both vertebral arteries were electrocauterized through the alar foramens located on the lateral surface of the atlas. 24 h later under sodium pentobarbital anesthesia (60 mg/kg, i.p.), the bilateral common carotid arteries (CCAs) were clamped with non-traumatic aneurysm clips for 10 min. Body temperature was monitored and maintained at 37 °C by an automatic heat controller desk.

In vitro electrophysiology

The electrophysiological recordings were carried out 8 days after the 4VO. The rats were decapitated, and vibratome-cut 350-μm coronal slices were prepared from the middle part of their dorsal hippocampi. The stimulating electrode, a concentric bipolar platinum-iridium electrode, was placed in the stratum radiatum near the border between the CA1 and CA2 subfields as to allow orthodromic stimulation of the Schaffer collateral/commissural pathway. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the stratum radiatum and stratum pyramidale. LTP of the Schaffer collateral-CA1 synaptic response was

induced by theta-burst stimulation (TBS) at 100% intensity of the test stimulus. After the LTP induction, the increased fEPSPs were recorded during at least a 60-min follow-up period. When the amplitudes were generally stable, their mean value was determined as the 10-min-long baseline. Input-output (I/O) curves were created to measure the basal Glu-ergic synaptic transmission.

In vivo electrophysiology

Electrocorticogram (ECoG) recordings were carried out on Wistar rats under sodium pentobarbital anesthesia (60 mg/kg i.p.). The skull was opened by drilling at the stereotaxic coordinates of the primary motor and somatosensory cortices. Four epidurally positioned silver-ball electrodes with an eight-channel electroencephalograph were applied to record electrocortical activity against a common central reference placed on the median line 5 mm anterior to the bregma. After the recording of a 15-min baseline, 10 min of 4VO was performed and the electrocortical activity was monitored for a further 50 min after the global cerebral ischemia. The sampling frequency was 200 Hz. We analyzed the burst suppression ratio (BSR), the number of spikes appearing 10-15 min after the start of reperfusion following the 10-min 4VO, the frequency composition of the ECoG after the FB under ischemic conditions, and the effects of OxAc on these parameters.

Data analysis

BSR analysis

Intermittent cortical activity observed after the ischemic insults, was quantified by estimating the BSR defined as the percentage of time spent in suppression. We applied the method of Vijn and Sneyd (1998) for burst identification. The resolution of the BSR estimation was 10 s. The absolute value of the 5 min baseline period of every channel was used to determine BSR repeatedly with a decreasing voltage threshold and with 200 ms as the minimum allowed BS duration. A threshold with a BSR value of <5% (20-25 μV on average) was used to monitor the changes in BSR after the first burst (FB) following transient global cerebral ischemia.

Threshold-crossing event detection

After global cerebral ischemia, 10-15 min of isoelectric ECoG can be measured. After the FB a post-ischemic burst period follows the isoelectric period which is approximately 20 min long as it is characterized by a higher rate of firing. The number of threshold-crossing spikes in every 5 min was determined in the 30-min-long period after the FB and divided by the number of spikes counted in the period between 30-35 min after the FB.

Spectral density estimation

To convert ECoG data from time-domain to frequency-domain and to compute the discrete Fourier transform and its squared magnitude, the method of Welch was used to calculate periodograms in order to estimate the power of ECoG at different frequencies. Every first 30 s of every 5-min period of ECoG after the FB was measured. The segment length comprised 300 data points with 50% overlap.

Statistical analysis

For LTP analysis, maximum fEPSP amplitudes were expressed as percentages of that of the baseline. The data were tested with the Shapiro–Wilk normality test and the Levene test. Since the results of the normality tests were significant and the requirement for the homogeneity of variances was not satisfied, further analysis was carried out with the non-parametric Kruskal–Wallis test and the pairwise comparisons of the groups with Mann-Whitney U-test. Analysis of the input–output curves was carried out with pairwise comparisons of the groups, related to certain input stimulus were also achieved with the Kruskal–Wallis ANOVA. The effects of the different rats were used as random effects and the different treatments were used as fixed effects in the mixed effect linear model. The

normal distribution of the BSR data could not be presumed using the Shapiro–Wilk normality test and the Levene test did not demonstrate an equality of variances. Therefore Mann-Whitney U-test as a non-parametric test on two independent samples was chosen for statistical analysis of the BSR data. The normal distribution of the threshold-crossing event count data could be observed according to the Shapiro–Wilk normality test. The one-sample T-test was used to determine the significance of difference between the two groups. In every case levels of significance were * = $p \leq 0,05$; ** = $p \leq 0,01$; *** = $p \leq 0,001$.

Histology

The animals were transcardially perfused with 4 % paraformaldehyde. In the 4VO experiments 20 μm thick coronal sections were made in the stereotaxic coordinates of -2,3 mm to -4,3 mm from bregma.

Fluro Jade-C staining

The brains were removed, and postfixed overnight in paraformaldehyde at 4°C. To reveal neuronal degeneration, staining with Fluoro Jade C (FJ-C) was applied, which has a high affinity for the entire degenerating neuron, including cell body, dendrites, axon and axon terminals. The slides were cover-slipped with Fluoromount and subsequently protected from direct light.

Cresyl violet

Morphological properties of CA1 pyramids were assessed by means of conventional cresyl violet nuclear staining.

Evans Blue staining

For Evans blue staining, permanent 4VO was carried out. Immediately after cauterization of the vertebral arteries both CCAs were exposed and two ligatures were placed upon each. Between the two ligatures, the arteries were severed in order to ensure interruption of the carotid flow. For the detection of the cerebral blood flow occurring during 4VO, the animals were perfused transcardially with 50 ml of 3% buffered Evans blue dye. The brains were removed, and postfixed overnight in 4% paraformaldehyde at 4°C. Coronal sections (20 μm) were obtained at the level corresponding to ECoG recording electrodes and were mounted on slides coated with 2% gelatin. The slides were cover-slipped with Fluoromount and subsequently protected from direct light.

Drug administration

Oxaloacetate

Oxaloacetate (OxAc) was dissolved in TBS buffer and adjusted to pH 7.3 with NaOH (10 N). The total volume administered was 1 ml. OxAc was injected i.v. during 15 min immediately after the end of the 10-min 4VO. Low-dose OxAc group received a dose of 4 mg/100 g bw while the high-dose OxAc group received 20 mg/ 100 g bw OxAc.

Dehydroepiandrosterone (DHEA)

DHEA was dissolved in 97% abs. EtOH and administered i.p. in corn oil in a concentration of 10 mg/ml in a total volume of 0.5 ml and it was administered 24 h after the transient global cerebral ischemia, in a dose of 2 mg/100 g body weight.

Results

1. The analysis of our ECoG data showed that the cortical activity was not completely blocked in the SD rats during the 10-min-long 4VO, and there was no sign of a post-ischemic burst period either. In the Wistar rats, we recorded isoelectric ECoG, during the 10-min 4VO and during the first 10-15 min of the reperfusion phase followed by a 30-min post-ischemic burst period. The BSR analysis indicated that the proportion of isoelectric ECoG periods increased at the expense of continuous firing during the post-ischemic burst activity. 25 min after the FB, BSR% values in th Wistar rats were 40-50%. BSR% for the SD rats already decreased to 5-10% during the 10-min 4VO, and further decreased to 0% immediately after the start of the reperfusion phase. This means that the cortical firing became similar to that seen in the baseline period. The cortical activity was characterized by the number of ECoG spikes. The lack of the post-ischemic burst period was also confirmed by the changes in the number of ECoG spikes after 4VO in the SD animals. In the Wistar rats we measured a more than 300% increase in the number of ECoG spikes in the 15th min following the FB. The ratio of the frequency components changed after the 10-min 4VO in both strains, but the frequency composition of the ECoG in the SD rats returned to that of the baseline in the 30th min after the FB. In the Wistar animals, the spectral density in the 30th min after the FB and that of the baseline were strongly different. The Fluoro Jade and cresyl violet hippocampal stainings revealed a pronounced difference in the magnitude of cell loss after 4VO. The SD rats displayed a much smaller amount of degenerated or dead cells in their hippocampal CA1 region after 10 min of 4VO. The neurons in the cortical areas of the ECoG recordings exhibited a much more intense Evans blue labeling after the permanent 4VO followed by the transcardial perfusion with the fluorescent dye in the SD animals than in the Wistar rats. The cerebral areas were less perfused in the Wistar rats during permanent 4VO. The capillaries of the cerebral cortex were still filled with blood after transcardial perfusion of the Wistar animals with phosphate buffer. The blood was washed out from the cerebral cortex of the SD rats and it demonstrated a strong labeling with Evans blue. Cortical neurons demonstrated a strong Evans blue staining, since they had the occasion to take up the Evans blue labeled serum albumin getting through the blood-brain-barrier disrupted by the ischemic conditions. In order to design a potent neuroprotective strategy, we use, an ischemic rat model which involves a sufficient amount of degenerated and dead cells to allow investigations of the neuroprotective effects of potential neuroprotectants. Our results we suggested that Wistar strain was an ideal animal for the 4VO surgery.

2. The neuroprotective effects of low- and high-dose OxAc administration, DHEA adminisration and combined treatment were examined on coronal hippocampal brain slices from the brains of 4VO animals 8 days after the ischemic insult. In the sham-operated animals, LTP induction by theta-burst stimulation resulted in a 150% increase of the fEPSP amplitudes relative to the baseline. In the 4VO group, the fEPSPs recorded following the LTP induction attained a maximum increment of only 128% and it gradually decreased during the 60-min follow-up to 109%. I.v. low-dose OxAc did not increase the fEPSP amplitudes after the theta-burst stimulation in comparison with the 4VO group. Administration of the high-dose OxAc resulted in a significant 133% growth of the induced LTP. DHEA treatment proved to cause an increasing tendency compared to the 4VO group, which was not significant during the 60-min recording. In contrast, the combined treatment was successful in restoring the impaired LTP to approximately 144%, i.e. nearly the value for the sham-operated group, and it was stable during the 60-min post-theta recording. There was no significant difference between the results of the sham-operated and the combined treated group. I/O curves were recorded to investigate the condition of the basal Glu-ergic transmission. Curves were established by plotting the fEPSP amplitude against different intensities of the test pulse ranging from 10 to 100 μA. The transient global cerebral ischemia affected the functioning of the Glu-ergic transmission machinery adversely and reduced the maximum measurable fEPSP from 3 mV to 1 mV. High-dose OxAc and DHEA separately caused a 150% increase of the fEPSP amplitudes in respect of the 4VO group, to approximately 1.5 mV. Combined treatment with high-dose OxAc and DHEA restored the impaired basal Glu-ergic transmission to a maximum value of 2.8 mV. Neuroprotective effects of OxAc on the early excitotoxic phase were investigated after 10 min of 4VO. The

results of the BSR analysis of the ECoG data showed the appearance of a 20-min post-ischemic burst period after the FB. In this burst period, the proportion of isoelectric ECoG increased compared to that in the firing periods. BSR% was 40-50% after the 25-min period following the FB. Administration of OxAc in the high dose (20 mg/100 g bw) resulted in a decrease of BSR%. Furthermore, we determined the spike count in the 30-min period after the FB. The number of spikes decreased in the post-ischemic burst phase of the OxAc group. Thus, we assumed that OxAc could decrease the Glu concentration of the ISF by expressing its Glu scavenger effect. OxAc exhibited neuroprotection in changing the ratio of the ECoG frequency components. In the control group, the spectral density was significantly different in the baseline and at 30 min after the FB. In the OxAc group, the spectral density curves in the identical periods were similar. The ratio of the frequency components returned to that of the baseline.

Conclusions

The use of different rat strains (e.g. Wistar vs. SD) can be a source of considerable variability in the results of acute experiments on global ischemia and it is important that the laboratory rats used in such experiments should be carefully chosen. We therefore studied Wistar and SD rats to determine whether there were interstrain differences in the responses to global ischemia elicited by 4VO in terms of the acute spontaneous electrical activity of the cerebral cortex and the consequent neurodegeneration. We saw striking differences between the two rat strains in the BSR% values, in the number of post-ischemic ECoG spikes, in the pattern of the first 20 s after FB, and in the proportion of the frequency components during and after 4VO. FJ-C and cresyl violet stainings demonstrated sporadic cell death in the 4VO SD and a much higher magnitude of apoptotic cell death in the hippocampus of the 4VO Wistar rats. We can conclude that in contrast with the SD strain, the Wistar rat is adequate 4VO model for testing neuroprotective strategies.

A combined treatment with endogenous compounds was tested as a promising new neuroprotective strategy in the hippocampal CA1 region. DHEA treatment itself resulted in only a slight increase in the induced LTP level. OxAc was significantly neuroprotective. Combined treatment resulted in strong neuroprotection and there was no significant difference between the results of the negative control and the results of the combined treated group. Our *in vivo* experiments revealed that the neuroprotective effects of OxAc can be seen after 30 min. It was sufficient to administer OxAc once, immediately after the ischemic insult, in order to achieve neuroprotection. DHEA is able to downregulate late ischemic events such as inflammatory processes. DHEA additionally has many neuroprotective activities which also contribute to decreases in neuronal and functional loss in the affected area. Both compounds are endogenous and occur naturally in the body. This combined treatment could be tested more safely in human trials than could synthetic exogenous molecules. Combined treatment could be a promising new neuroprotective strategy in the clinical care of stroke.

Publication directly related to the PhD thesis

Neuroscience 10.1016/j.neuroscience 2012.10.042 **impact factor: 3.380**

Fundamental interstrain differences in cortical activity between Wistar and Sprague-Dawley rats during global ischemia

Fuzik J; Gellért L.; Oláh G.; Kocsis K.; Knapp L.; Nagy D., Z Kincses T.Z., Kis Zs., Farkas T., Toldi J.

Publications not directly related to the PhD thesis

Neuropharmacology 2011;61:1026-32 **impact factor: 4.677**

Kainate postconditioning restores LTP in ischemic hippocampal CA1: Onset-dependent second pathophysiological stress

Nagy D, Kocsis K, **Fuzik** J, Marosi M, Kis Z, Teichberg VI, Toldi J, Farkas T.

European Journal of Pharmacology 2011;667:182-7 **impact factor: 2.587**

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Oxaloacetate restores the long-term potentiation impaired in rat hippocampus CA1 region by 2-vessel occlusion

Marosi M, **Fuzik J**, Nagy D, Rákos G, Kis Z, Vécsei L, Toldi J, Ruban-Matuzani A,Teichberg VI, Farkas T.

Cumulative impact factor: 13.231