Study of different neuroprotective strategies on ischaemic rat models

Summary of Ph.D. thesis

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Introduction

Focal and transient global ischaemia, after stroke or in atherosclerosis or vasculitis in humans or be induced experimentally in animals, elicits selective, delayed neuronal death. Disorders of the cerebral circulation are the causes of or factors contributing to numerous neurological and psychiatric illnesses. A sudden disruption of the blood supply to distinct brain regions leads to stroke, while a moderate but persistent reduction in regional cerebral blood flow (CBF) compromises memory processes and contributes to the development and progression of dementia. If the duration of the ischaemia is short, neuronal damage occurs only in vulnerable areas.

In acute ischaemic insults, glutamate (Glu) causes irreversible neuronal damage, which is generally observed at the histological level by the presence of a cortical infarction, and at the electrophysiological level by the correlated loss of somatosensory evoked potentials (SEPs) cortical disinhibition, and peri-infarct depolarizations. It is widely accepted that activation of the excitatory amino acid receptors plays an important role in neuronal death in stroke. It has recently been reported that Glu-induced excitotoxicity and a cellular calcium overload are among the key factors of cell death in brain ischaemia, especially in the gray matter. By definition, excitotoxicity is a result of overexcitation of the Glu receptors.

Numerous neuroprotectants are tested in ischaemic animal models to reduce infarction and improve the neurological status, but unfortunatelly only few reach the clinics. Since the most of the functional consequences of ischaemia can be blocked by the administration of Glu receptor antagonists, one can expect that the elimination of the excess Glu present in the brain interstitial fluids upon ischaemia will be of beneficial value. Recently, blood Glu scavenging has been shown to cause an increased brain-to-blood Glu efflux, to eliminate the excess Glu in the brain interstitial fluids, and to provide neuroprotection after a traumatic brain injury. The intravenous administration of oxaloacetate (OxAc) or pyruvate, which activates the blood-resident glutamate–oxaloacetate transaminase and glutamate–pyruvate transaminase, respectively, leads to blood Glu scavenging by the transamination of Glu into 2-ketoglutarate, as a result of an accelerated efflux of the excess brain Glu into the blood, and neuroprotection is achieved. On these premises, we tested here the prediction that the intravenous administration of the blood Glu scavenger OxAc should cause neuroprotection after an acute ischaemic insult, and improve both histological and functional manifestations of ischaemia, as evaluated by Fluoro-Jade B (FJB)-staining histology and the measurement of SEPs. As acute

ischaemic insult, we elected to use the photothrombosis model. During photothrombosis, a photosensitive dye circulating through the cerebral vasculature is exposed to an externally applied light beam. Light exposure generates highly reactive oxygen radicals in the local blood stream. The reactive oxygen species disrupt the capillary endothelium, causing microvascular platelet aggregation and disruption of the blood–brain barrier.

2VO (only the bilateral common carotid arteries are clamped) reduces the blood flow to one-third in rats, with only a slight malfunction in synaptic plasticity. Through glutamatergic synapses, long-term potentiation (LTP) involved cellular mechanisms of learning and memory expresses mainly dendritic spines in the hippocampus and cortex. Any LTP malfunction in the ischaemic hippocampus is paralleled by changes in dendritic spine density through remodeling of spines.

A new and endogenous neuroprotective strategy is ischaemic postconditioning, which has undergone renaissance during the last 5-10 years of intensive research. Ischaemic postconditioning in the brain involves several repeated cycles of brief reperfusion and reocclusion of the common carotid artery after the onset of full reperfusion. Postconditioning can also be elicited by the single post-ischaemic administration of many pharmaceuticals (second pathophysiological stress). Kainic acid (KA), obtained first from the red alga *Digenea simplex*, is a potent agonist of the kainate class of ionotropic glutamatergic receptors, and hence a potent neuroexcitant through excitotoxicity.

We have tested whether KA treatment is suitable for pharmacological postconditioning after incomplete global ischaemia. In particular, we determined the optimum application timepoint (therapeutic window) for postconditioning during the 72-h post-ischaemic period after 2VO, the changes in spine density in the hippocampal CA1 region, the basal glutamatergic synaptic transmission and the LTP.

The aims of the study

1. The aims of our research group were to study the effects of photochemically induced focal ischaemia in rat cortex. To determine the functional and histological consequences we measured the changes in amplitudes of evoked potencials, the volume of the infarct size and the number of the degenerating neurons.

2. We tested here the prediction that the intravenous administration of the blood glutamate scavenger oxaloacetate should cause neuroprotection after an acute ischaemic insult, and improve both histological and functional manifestations of ischaemia.

3. After performing 30 minutes long two vessel-occlusion (2VO) we could not detect any morphological changes by the classical stainings (Fluoro-Jade B, S-100 and Nissl staining) even on the highly vulnerable brain areas either. Besides *in vitro* electrophysiological measurements we were to optimize a sufficiantly sensitive morphological method to visualize the synaptical consequences of global cerebral hypoperfusion.

4. We have tested whether kainate treatment is suitable for pharmacological postconditioning after incomplete global ischaemia. In particular, we determined the optimum application timepoint (therapeutic window) for postconditioning during the 72-h post-ischaemic period after 2VO, the changes in spine density in the hippocampal CA1 region, the basal glutamatergic synaptic transmission and LTP.

Materials and methods

Animals

The study was performed on adult male Wistar rats (n=110, 220–300 g) maintained under controlled environmental conditions at a temperature of 22±2 °C and a 12-h light/dark cycle. Food and water were available ad libitum. The local Animal Ethics Committee had approved all the experiments. The care and use of the experimental animals were in accordance with the 86/609/EEC directive.

Focal ischaemia model

Photothrombotic lesion

The cortical photothrombotic lesion was induced by the tail vein injection of Rose Bengal (3 mg/100 g) and cold light exposure. The illumination (through the intact skull, with a cold light source) was started just after the Rose Bengal injection, and lasted for 20 min. Four hours later, the animals were transcardially perfused with 4% paraformaldehyde in 0.1 Mphosphate buffer.

Histological staining: Fluoro-Jade B

The brains were removed and postfixed overnight in fixative solution. Coronal sections (36 μm) were cut with a freezing microtome. Fluoro-Jade B (FJ-B) is a fluorochrome that stains degenerating neurons with high selectivity. The sections were observed under a microscope in fluorescent light at an excitation wavelength of 470–490 nm and an emission wavelength of 520 nm. The volume of the hemispheric lesion and the number of FJB-positive cells were calculated for each animal.

In vivo electrophysiology

SEPs induced in the contralateral primary somatosensory cortex were recorded from the surface of the skull with the aid of a silver electrode. The right whisker pad was stimulated electrically with a bipolar needle electrode (0.1 Hz, 0.3 ms duration and 4–6 V) to evoke visible whisker movements. Seven recording points were situated along a virtual line parallel to and 5 mm laterally from the midline, including the SSI, i.e., at the points 0, -1, -2, -3, -4, -5, and -6 mm frontal to the bregma in all the animals. Control series were recorded in each group prior to induction of the photothrombotic lesion. The following recording period started

1 h later, after the induction of the photothrombotic lesion, while the successive series of recordings started at 2, 3, and 4 h postinsult.

Oxaloacetate treatment

In the OxAc-treated group, the animals received OxAc solution (1.2 mg/100 g, i.v., pH 7.2–7.4) at a constant rate through the tail vein, during a 30-min period. This dose was used because it was shown in previous studies to cause both blood Glu scavenging and neuroprotection. Oxaloacetate was dissolved in 1M sodium hydroxide diluted with physiological saline and adjusted with 10 M sodium hydroxide to pH 7.4. The OxAc injection started just after the cold light exposure.

Statistical Analysis

To test the statistical significance of differences between the responses at the *punctum maximum* in the different groups, as a function of time, one-way ANOVA followed by the post hoc Bonferroni test was applied. When the EPs were compared inside the groups at the various coordinates, the paired-sample t-test was applied. A P value of\0.05 was considered significant.

Transient global hypoperfusion model

Preparation of hipoperfusion model – Two vessel occlusion (2VO)

Before the induction of transient cerebral ischaemia, the rats were anaesthetized with 4% chloral-hydrate (i.p.) and the body temperature was maintained at 37±0.5 °C throughout the procedure. The common carotid artery were isolated and clamped with non-traumatic aneurysm clips. The sham-operated control rats underwent the same procedure, but without common carotid artery occlusion. The carotid artery blood flow was reperfused by releasing the clips following the 30-min occlusion.

In vitro electrophysiology

The electrophysiological recordings were conducted 3 days after the termination of the bilateral common carotid artery occlusion. The rats were decapitated, and vibratome-cut coronal slices (400 μm) were prepared from the middle part of their hippocampi in an ice-cold

artificial cerebrospinal solution (aCSF), saturated with 95% O_2 and 5% CO_2. The stimulating electrode was placed in the stratum radiatum in CA1 regions to allow orthodromic stimulation of the Schaffer collateral/commissural pathway. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the stratum radiatum with a 1–2-MOhm resistance glass microelectrode. LTP of the Schaffer collateral-CA1 synaptic response was induced by high-frequency stimulation (0.2-ms pulses delivered at 100 Hz for 5 s), and after the HFS the fEPSPs were recorded for at least a further 60 min. The fEPSPs were monitored for 40–60 min before conditioning stimulation until the amplitudes were generally stable, and their mean value was determined as the 10-min-long baseline.

Golgi-Cox staining

In order to determine the number of apical dendritic spines in the hippocampal CA1region in the control, 2VO and KA-postconditioned animals, we used the Golgi techniques. The animals were decapitated, and the brains were rapidly, but carefully removed from the skull. Approximately 3-mm sagittal blocks including the whole hippocampi were placed in Golgi-Cox solution. When the impregnation was complete 100-μm sagittal sections were cut with a vibratome. The clear Golgi sections were studied by light microscopic stereology, using oil immersion objectives. The spine density of the proximal apical dendrite area (100-200 μm from the soma) was analysed. The whole process of measurement of the spine number and density was performed separately by three experimenters blind to the analysis.

Kainate treatment

In the postconditioned groups the reperfusion period was always 72 h. For KA treatment 5 mg/kg dose (a result of a personal communication with J. Burda) was used intraperitoneally to obtain the neuroprotective effects. The kainate was dissolved in saline solution and the volume was 1 ml.

Statistical analysis

In the spine density analysis of the data for the various experimental groups parametric t-tests for two independent samples were used. For LTP measurements, the fEPSP amplitudes were normalized to the means of the 10-min pre-HFS control data. As normal distribution of the data could not be presumed and the Levene test did not demonstrate equality of variances, a

non-parametric test on two independent samples was chosen for the statistical analysis of the LTP data (Mann-Whitney U-test). For the statistical analysis of IO curves, the Kruskal-Wallis test was chosen (Fig. 5). A P value of ≤ 0.05 was considered significant. SPSS10.0 for Windows software was utilized.

Results

Focal ischaemia model

In vivo electrophysiology

SEPs were recorded at seven points with maximal amplitudes (*punctum maximum*) obtained at the coordinates frontal: -3 mm, lateral: 5 mm with respect to the bregma. The amplitudes of these SEPs facilitated slightly in the course of the 4-h experiments, during which repeated stimulations were carried out at 1-h intervals. The above experiments were repeated on rats subjected to a photothrombotic lesion. The photothrombotic lesion resulted in an immediate reduction in the amplitude of the EPs measured at the *punctum maximum* and at all the other points. Rats treated with OxAc after induction of the photothrombotic lesion displayed less profound decreases in the EP amplitudes at the punctum maximum than those in the lesioned rats, and the EPs exhibited a very distinct recovery, reaching 80–82% of the control levels at 3–4 h post-illumination. Normalization of the EP amplitudes at the punctum maximum to those of the control EPs measured during pre-illumination revealed that the extent of recovery of the EP amplitudes measured at 3–4 h post-illumination in the lesioned and OxAc-treated rats was much higher than that in the lesioned and untreated rats. Comparison of the EP amplitudes of the sham-operated (control) animals with those in the lesioned and lesioned + OxAc-treated groups indicated that the patterns of changes at all the recorded points were similar to those observed at the punctum maximum. To summarize, normalization of the amplitudes measured at the various coordinates to those measured at the punctum maximum demonstrated that the response amplitudes gradually became smaller in both the rostral and caudal directions throughout the experiment, and in the lesioned group, but not in the lesioned + OxAc-treated group, were significantly different from those in the sham-operated control group.

Fluoro-Jade B staining

FJB is a polyanionic fluorescein derivative which sensitively and specifically binds to damaged neurons, with increased contrast and resolution during acute neuronal stress. One can observe very distinct FJB staining 9nt he lesioned untreated group indicative of the presence of neuronal cell damage. Serial analysis of the FJB staining 9nt he contiguous brain sections carried out to determine the lesion volume revealed that the treatment with OxAc reduced the volume of the thrombotic lesion by about 30%. At higher resolution, the counting of the FJB- positive cells confirmed these results and showed that the OxAc treatment reduced the number of stained cells by about 30%.

Global ischaemia model

Golgi-Cox staining

The Golgi-Cox staining method labelled a subset of neurons in all parts of the hippocampus. In the control group, the spine density was 182.07±3.25/100 μm. In the 2VO animals the mild, incomplete ischaemia resulted in a marked decrease in spine density (84.95±2.23/100 μm). With the aid of prior electrophysiological experiments (LTP measurements), the ideal timepoint for KA-postconditioning was determined, which proved to be 48 h after 2VO. For examination the consequences of postconditioning as concerns the spine density, measurements were subsequently made only at this single timepoint. As a result of KA-postconditioning, by 48 h after ischaemia the spine density had virtually recovered to the control level (171.96±2.58/100 μm vs 182.07±3.25/100 μm) in parallel with restoration of the synaptic plasticity.

In vitro electrophysiological recordings

The HFS of Schaffer collaterals resulted in a stable 45% increase in fEPSP amplitudes in the controls, which remained stable throughout the 1-h recording period. A 30-min 2VO, 72 h prior to LTP induction, resulted in a lesser increase in amplitudes (15%), with a constant decay in time. KA-postconditioning applied 24 h after ischaemia had a worsening effect on the LTP function (113%). When KA postconditioning was applied immediately after 2VO, the fEPSP amplitude increase was least, at only 112%, and in this group no post-tetanic potentiation (PTP) was detected. An LTP function recovery was seen only when KA treatment was applied 48 h after ischaemia (delayed postconditioning), clearly demonstrating

the onset dependence of the postconditioning. In this group, the post-HFS values were at close to the control levels throughout the 1-h follow-up period. In both these early postconditioned groups, the amplitudes had reached the pre-HFS levels by the end of the 1-h follow-up period.

The basal synaptic properties were also tested to evaluate how the various experimental interventions impaired the Schaffer collateral-CA1 synaptic transmission. IO curves were established by plotting the fEPSP amplitude against various intensities of the test pulse, ranging from 5 to 100 μA in 5 μA steps. It is clearly seen that there was a marked difference between the IO curves of the control and the 2VO groups, implying that the basal functions of the pyramidal cells and synapses were affected by the 2VO. However, the KA-postconditioned group demonstrated normal input-output values.

Discussion

Focal cerebral ischaemia induces a complex series of events that damage the brain cells. The very early events include massive depolarization due to Glu release and the loss of SEPs. In photothrombotic ischaemia, repetitive episodes of cortical spreading depression lasting up to 3 h are observed and are accompanied by a massive rise in extracellular Glu level lasting up to 5 h post-illumination. The role of Glu is clearly critical since the anatomical correlates of the photothrombotic ischaemia can be significantly attenuated following the administration of Glu receptor antagonists. This study was motivated by the expectation that the elimination of excess Glu after ischaemia would have a positive impact on both the anatomical and functional correlates of ischaemia. First of all, we predicted that the volume of the ischaemic lesion would be decreased after OxAc administration, because of the increased brain-to-blood Glu efflux. Indeed, the administration of the Glu scavenger OxAc at a dose shown in previous studies to cause both blood Glu scavenging and neuroprotection resulted in a reduction in the volume of the ischaemia-induced cortical damage. This effect could be observed as early as 4 h after the intervention. Moreover, we expected that the photothrombotic lesion would result in decreased amplitudes of the SEPs as these are early consequences of ischaemia. We also anticipated that OxAc would attenuate the decreases in the EP amplitudes. The results we obtained clearly fulfil these expectations since the administration of the blood Glu scavenger OxAc after the photothrombotic lesion attenuated both the volume of the infarct and the decreases in the amplitudes of the EPs. Thus, as predicted from its blood Glu-scavenging

activity and its ability to increase the efflux of excess Glu from the brain into the blood.

A great wealth of experimental data strengthen the assumption that remodelling and stabilization of the spine synapses of the pyramidal cells are features of the cellular mechanism by which LTP and memory processes occur. Pyramidal cells receive almost all their glutamatergic excitatory input through their spine synapses, and the changes in the spine density and spine synapses can therefore greatly influence the excitability and responsiveness of these cells. Under pathological circumstances, as in the ischaemic brain, the longer dendritic spines at the network level can isolate the soma biochemically and electrically from the local exitotoxic effects, preventing the initiation of apoptotic processes. Spines can buffer the elevated Ca^{2+}-levels during excitotoxicity. The dendritic plasticity in the post-ischaemic brain is the early adaptive response by which affected neurons can fend off pathological processes, such as excitoxicity, increased Ca^{2+} influx, and spontaneous spreading depolarizations. Incomplete forebrain ischaemia, e.g. that caused by 2VO, does not lead to cell death, thus, the change in dendritic plasticity alone may be responsible for the reduction of LTP in the CA1 region. KA is a neuroexcitatory and neurotoxic substance, its administration locally or systemically induces widespread neurodegeneration in the brain, the most intense being in the hippocampus, accompanied by the loss of the principal cells in the CA1, CA3 and hilar regions. However, the administration of KA also triggers neurogenesis in the dentate gyrus. Whether or not a cell survives or undergoes apoptosis is dependent on the activation of these MAPKs. CaMKII plays a key role in mediating cell death following hypoxia/hypoglycaemia, and displays an elevated phosphorylated level following KA treatment. In our experiments, the applied KA concentration was low (5 mg/kg, i.p.) and, although the above-mentioned processes were not studied and hence can not be excluded, our results appear to be rather related to the “more physiological” activation of kainate receptors. In the CA1, the pharmacological activation of GLu5R-containing KA receptors strongly depresses glutamatergic transmission through the action of the presynaptic receptors via G-protein-coupled signalling. Moreover, the sustained activation of GluR5 receptors causes a specific and lasting increase in the number of glutamatergic synapses in area CA1, dependent on PKC activation. Our results demonstrate that, while KA treatment hampers synaptic plasticity in the adult hippocampus under normal circumstances, in the ischaemic hippocampus KA is able to restore the injured synaptic plasticity within its therapeutic window through the restoration of the control level of spine density and normal-like glutamatergic synaptic transmission. Delayed postconditioning is based on the synthesis of

proteins and is able to reverse the ischaemia-induced apoptosis-like process of delayed neuronal death 48 h after ischaemia. Changes induced by postconditioning include the prevention of cytochrome c and MnSOD release into the cytoplasm and the decreases in activity of caspase-3 and iNOS and the substantial drop in glutamate concentration. Our experiments have demonstrated that KA treatment induces spine-genesis in the adult hippocampal CA1 subfield after incomplete global ischaemia, which results in a normal-like functioning neural network.

Conclusion

In conclusion, the attenuation of the anatomical and electrophysiological correlates of the brain ischaemia produced by OxAc are in line with the neurological improvements it causes in closed head injuries and provide additional evidence for the neuroprotective activity of OxAc.

KA can be used as a pharmacological postconditioner only at the second post-ischaemic day, revealing a very specific therapeutic window. This is in agreement with previous studies, which involved either ischaemic or pharmacological postconditioning with therapeutic windows ranging from 6 h up to 2 days.

Original publications directly related to the PhD thesis

Cellular and Molecular Neurobiology (2009) Sep;29(6-7):827-35. *(Impact faktor: 2,483)*
Oxaloacetate decreases the infarct size and attenuates the reduction in evoked responses after photothrombotic focal ischemia in the rat cortex
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Cumulative impact faktor: 13,533