

PH.D. THESIS

NEUROPROTECTIVE STRATEGIES AGAINST ISCHEMIC BRAIN INJURY

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Introduction

Transient global ischemia, which may arise during cardiac arrest and surgery 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 ischemia is short, neuronal damage occurs only in vulnerable areas. The pyramidal neurons in the hippocampal CA1 region are particularly vulnerable. Other neurons, such as the hippocampal CA3 neurons, cortical pyramidal cells are less ischemia-vulnerable.

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 glutamate (Glu)-induced excitotoxicity and a cellular calcium overload are among the key factors of cell death in brain ischemia, especially in the gray matter. By definition, excitotoxicity is a result of overexcitation of the Glu receptors. The hippocampus, where cognitive processes are believed to depend on changes in synaptic efficiency, is a brain region extremely rich in glutamatergic synapses and sensitive to Glu excitotoxicity. These glutamatergic synapses underlie the process of long-term potentiation (LTP), which is a leading candidate mechanism in learning and memory. In turn, neuroprotective strategies have utilized antagonists of the Glu receptors to prevent excitotoxic neuronal loss. Long-term potentiation (LTP), also mediated by Glu receptors, is a model of neuronal plasticity. Accordingly, ischemia may likewise impair physiological forms of synaptic plasticity, such as activity-dependent LTP.

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Kynurenic acid (KYNA) is one of the few known endogenous N-methyl-D-aspartate (NMDA) receptor inhibitors. Experimental data and theoretical considerations suggest that KYNA or its metabolic precursor, L-kynurenine (KYN), may be of therapeutic value in neurodegenerative disorders. The use of KYNA as a neuroprotective agent can be excluded because it is barely able to cross the blood-brain barrier (BBB), whereas KYN is transported across the BBB much more readily by a neutral amino acid carrier.

The excess Glu, which causes neuronal death via excitotoxicity, is normally controlled by members of a family of Na⁺-dependent Glu transporters located on nerve terminals and astrocytes. By pumping Glu, they guarantee the presence of Glu in brain fluids at levels at which it exerts neither excitotoxic nor unsolicited excitatory effects. Glu transporters located on the brain vasculature may also play an important role in controlling extracellular Glu levels via a brain-to blood Glu efflux. The precise localization and properties of the Glu transporters on the brain microvasculature have not yet been completely established. Light and electron microscopy studies have clearly shown that the Glu transporters EAAT1 and EAAT2 are located on the astrocytic endfeet rather than on the capillary endothelium. Nonetheless, O'Kane *et al.* have presented evidence of the presence of EAAT1, EAAT2 and EAAT3 on the abluminal membrane of bovine brain capillary endothelial cells. In agreement with these findings, by means of western blotting Teichberg *et al.* (2008) demonstrated the presence of these Glu transporters in porcine brain capillary endothelial cells. At any event, the brain-to-blood Glu efflux mediated by these transporters is fast and greatly enhanced by the blood Glu scavengers oxaloacetate and pyruvate which, upon intravenous administration activate the blood-resident glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase, respectively, causing Glu deamination into α -ketoglutarate. The scavenging of blood Glu increases the driving force for a brain-to-blood Glu efflux and leads to a decrease in the excess Glu present in the brain extracellular fluids.

The aim of the study

The aim of the present study was to reveal whether KYN can rescue the CA1 neurons in the four-vessel occlusion (4VO) model in the rat, which is a species more widely used than the gerbil in the course of ischemic studies. KYN was administered together with probenecid (PROB), an organic acid transporter inhibitor, in order to facilitate the brain penetration of KYN.

In the second series of experiments we tested the hypothesis that the intravenous administration of a blood glutamate scavenger, oxaloacetate, administered immediately after a 30-min period of incomplet forebrain ischemia (two wessel occlusion, 2VO), helps the brain retain its synaptic plasticity.

Materials and methods

Animals

The study was performed on adult male Wistar rats (n=101, 230–300 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.

Global cerebral ischemia – Four-vessel occlusion (4VO)

4VO was carried out as described previously. In brief, the rats were anesthetized with Nembutal. Both vertebral arteries were occluded by cauterization. The wounds were closed, and the animals were allowed to recover for 24 h. On the following day, the animals were subjected to 10-min forebrain ischemia by bilateral occlusion of the carotid arteries with a non-traumatic clips under ether anesthesia. The body temperature was monitored, and maintained at 37 °C during the surgical procedures. Both vertebral arteries were cauterized, and both common carotid arteries were exposed but not occluded in the sham-operated animals. The rats used for histology were divided into 4 groups: sham-operated controls (SC group, n=5), 4VO animals (4VO group, n=7), KYN+PROB pretreated animals (KYN+PROB-4VO group, n=6) and KYN+PROB-posttreated animals (4VO-KYN+PROB group, n=7). The rats used for electrophysiology were divided into 3 groups: sham operated controls (SC group, n=6), 4VO animals (4VO group, n=6) and KYN+PROB-pretreated animals (KYN+PROB-4VO group, n=6). KYN (300 mg/kg, i.p.) and PROB (200 mg/kg, i.p.) were administered daily for 5 days: in the pretreated group, the first KYN+PROB administration preceded the 10-min carotid occlusion by 2 h; and the animals were treated at the same time on the next 4 days. In the posttreated group, the animals received the first KYN+PROB injection at the start of reperfusion. The remaining 4 injections were given at the same time on the next 4 days.

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

Before the induction of transient forebrain cerebral ischemia, 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 reperused by releasing the clips following the 30-min occlusion. Oxaloacetate was dissolved in 1M sodium hydroxide diluted with physiological saline and adjusted with 10 M sodium hydroxide to pH

7.4. Oxaloacetate was injected at the various doses tested in a total volume of 1.5 ml. The higher doses of oxaloacetate were selected as *Teichberg et al.* (2008) had been shown to reduce blood Glu levels effectively. The lower doses were selected in order to determine a threshold of efficacy. The drug was injected intravenously immediately after the end of the 30-min common carotid artery occlusion, when the reperfusion started (post-treatment). The drug administration lasted for 30 min. Control rats were injected with an equal volume of saline.

Histological staining: Fluoro-Jade B, S-100, NeuN and cresyl violet

The treated and control animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer 3 days after 2-vessel occlusion. The brains were removed and postfixed overnight in the same fixative. Coronal sections (36 μ m) 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.

Protein S-100 is one of a large family of calcium-binding proteins. In human cerebrovascular diseases, a significant correlation has been reported between the plasma concentration of the S100 protein and the volume of the cerebral infarct. S-100 was recently found to be a promising marker for central nervous system injury. Polyclonal rabbit antibodies to the cow S-100 protein (anti-S-100) were used to investigate the distributions of glial cells associated with S-100 after 2-vessel occlusion. The sections were incubated overnight at room temperature with the primary antibody directed against S-100 and were treated with the secondary antibody for 3 h at room temperature. Finally, the labelling was observed under a fluorescence microscope at an excitation wavelength of 530–550 nm and an emission wavelength of 590 nm.

For anti-neuronal nuclei (NeuN) immunohistochemistry, free floating sections were rinsed in TBS+ 0.4% TRITON. They were then preincubated with 1% NGS (normal goat serum) + TBS+ 0.4% TRITON for 1 h. Primary mouse (NeuN) antibody at a dilution of 1:2000 in 1% NGS, 0.4% TRITON, 0.05% Na-azide and TBS was then applied overnight at room temperature. After washing in TBS+ 0.4% TRITON (3 \times 10 min), sections were incubated with secondary CyTM3-conjugated donkey anti-mouse IgG (H+L) antibody for 3 h at room temperature. After further washing in TBS+ 0.4% TRITON (3 \times 10 min), sections were brush-mounted onto gelatin-subbed slides and, then dried in the air for 1 day in the dark.

The procedure of cresyl violet staining was performed as usual with a 1% filtered solution: the slide racks passed through the sequence of baths for the times indicated: 95% ethanol–15 min, 70% ethanol–1 min, 50% ethanol–1 min, distilled water–2 min, distilled water–1 min, cresyl violet stain–5 min, distilled water–1 min, 50% ethanol–1 min, 70% ethanol–2 min, 95% ethanol–2 min, 95% ethanol–a few dips, 100% ethanol–1 min, respectively.

In vitro electrophysiology (2VO and 4VO experiments)

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 slices were then transferred to a Haas-type recording chamber and incubated at room temperature for 1 h to allow the slices to recover in the solution used for recording. The flow rate was 1.5–2 ml/min and the experiments were performed at 34 °C. 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-M Ω resistance glass microelectrode that was filled with aCSF and connected to a neutralized, high input-impedance preamplifier with a high-pass filter set at 5 kHz. The test stimulus intensity was adjusted to between 30 and 60 μA . The fEPSPs were digitized, saved with a PC equipped with a Digidata 1200 interface and an Axoscope 10.0 recording system and analysed offline with Origin 6.0 software. 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) at 100% intensity of the test stimulus, and after the high-frequency stimulation 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. Paired-pulse facilitation was measured by using the intensity of the test stimulus with an interpulse interval of 25, 50 or 100 ms, and input-output curves were created to measure the basal glutamatergic synaptic function. Slices from the same animal were generally used for several tests, including LTP, input/output curves or paired-pulse facilitation. Each slice was subjected to only one particular test.

Results

4VO

Histology

In animals subjected to 10-min 4VO, severe neuronal damage was observed in the CA1 area of the hippocampus in both hemispheres 10 days after the intervention. The neuronal damage extended for millimeters in the anterior–posterior direction from – 3.14 mm relative to the bregma. In this injured region, numerous of the pyramidal cells in the CA1 region were FJ+ in each of the coronal sections, while those in the CA3 region and the dentate gyrus were not labeled. In accordance with this, the NeuN immunohistochemistry indicated the lack of intact cells in the CA1 region in the 4VO animals, but an intact CA3 region and dentate gyrus.

Kynurenine administration

KYN (300 mg/kg, i.p.) administered together with PROB (200 mg/kg, i.p.) caused a marked reduction in the number of damaged neurons. In the KYN-pretreated animals, injured neurons stained with FJ could be observed only sporadically in the CA1 area of the hippocampus (the same was true for the CA3 region and the dentate gyrus. Accordingly, NeuN immunohistochemistry gave the impression of a non-injured CA1 region (like the CA3 region and the dentate gyrus) in KYN-pretreated 4VO animals. KYN administration considerably decreased the number of injured neurons in the CA1 region. However, the decrease in the number of injured neurons was highly significant only in the pretreated group (KYN+PROB-4VO). The animals in the post-treated group (4VO-KYN+PROB) also exhibited a tendency to a reduction in the number of injured neurons, but this change was not significant. The NeuN immunohistochemistry supplemented these results: the number of non-injured cells was highest in the SC group, and lowest in the 4VO animals. Post-treatment with KYN (4VO-KYN+PROB) had hardly any effect, while in the 4VO animals which received KYN before ischemia (KYN+PROB-4VO) the number of intact cells was comparable to the control level. In short, the KYN+PROB pretreatment was able to reduce the proportion of damaged cells to 52% relative to the damaged cells induced by 4VO without KYN+PROB treatment. The KYN+PROB treatment after the 4VO intervention did not prove effective.

In vitro electrophysiology

First of all, we explored the basal synaptic properties of the fEPSPs in order to evaluate the ischemia-induced impairment of the Schaffer collateral-CA1 synaptic transmission. For this purpose, the IO curves were established by plotting the fEPSP amplitudes against various test

pulse intensities from 0 to 100 μ A. The IO curve for the 4VO animals was positioned below that for the controls (while the curve for the KYN+PROB-4VO animals was positioned above it). However, there was no significant difference between the IO curves in the three groups, implying that the basal functions of the registered pyramidal cells and synapses were not affected by complete ischemia. LTP was induced by HFS of the Schaffer collateral-CA1 synapses. 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 20-min-long baseline before LTP induction. In the SC group, the HFS caused a robust increase (130–140%) in the amplitude of the fEPSPs and this increase in amplitude remained at the elevated level during the 1-h registration period. The same conditioning protocol did not induce a significant, lasting increase of the fEPSPs in the majority of the 4VO animals. In this group, the elevation of the amplitudes was only transient; no LTP was observed. At the end of the registration period, the amplitudes had returned to the control level, or decreased below the baseline. The administration of KYN and PROB protected slices from the 4VO induced LTP impairment. KYN restored the fEPSP amplitudes to the control level, and these parameters were stable until 60 min after HFS (Sas *et al.*, 2008).

2VO

Histology

The histological analysis of the brain sections carried out 3 days after the transient brain hypoperfusion did not detect any changes in the CA1 region of the hippocampus. Neither Fluoro Jade B staining, nor S-100 immunohistochemistry nor cresyl violet staining revealed injuries/changes after the transient 2-vessel occlusion-induced hypoperfusion of the CA1 region.

In vitro electrophysiology

In the sham group, the high-frequency stimulation caused a robust increase (35–40%) in the amplitude of the fEPSPs, which remained at this elevated level throughout the 1-h registration period. The same conditioning protocol induced a significantly smaller increase in the amplitude of the fEPSPs in the 2-vessel occlusion group, observed 3 days after the start of the reperfusion. In this group, the high-frequency stimulation resulted in only a ~20% increase in amplitude, which remained at this level for about 30 min, and then gradually decreased. At the end of the registration period, the amplitudes were near the baseline (107–108%). The effect of oxaloacetate on the 2-vessel occlusion-induced LTP impairment was tested on hippocampal slices from animals exposed to intravenous oxaloacetate administered at doses

of 1.5 mmol or 2.7 μ mol. These doses were equally effective in protecting the LTP from the 2-vessel occlusion-induced impairment. The post-hypoperfusion administration of oxaloacetate resulted in a high-frequency stimulation-induced LTP comparable to that observed in the hippocampi of the control animals. Just after the high-frequency stimulation, a transient and statistically insignificant decrease in amplitudes occurred. The amplitudes then returned to a plateau at about 135–140%, similarly to the LTP observed in the hippocampi from the control rats. As compared with the 2-vessel occlusion group, the level of significance was reached by the control group at 7 min, by the 2-vessel occlusion high-dose oxaloacetate group at 7 min, and by the 2-vessel occlusion low-dose oxaloacetate group at the 33 min post-high-frequency stimulation. The protective effects of oxaloacetate on the LTP were dose-dependent. While maximum protective effects were observed for the doses of 1.5 mmol and 2.7 μ mol, 0.1 μ mol oxaloacetate was ineffective in preventing the hypoperfusion-induced LTP impairment. The basal synaptic properties were also tested to evaluate whether the 2-vessel occlusion impaired the Schaffer collateral-CA1 synaptic transmission. Input-output curves were established by plotting the fEPSP amplitude against various intensities of the test pulse ranging from 10 to 100 μ A. There is no distinguishable difference between the input-output curves of the control and the 2-vessel occlusion groups, implying that the basal functions of the pyramidal cells and synapses were not affected by the 2-vessel occlusion-induced hippocampal hypoperfusion. The paired-pulse ratio was also measured with interpulse intervals of 25, 50, 75 and 100 ms in both groups. The results strongly suggested that the transient incomplete ischemia did not impair the probability of presynaptic Glu release, since there was no significant difference between the groups (Marosi *et al.*, 2009).

Discussion

It is well known that various brain dysfunctions are closely related to a reduction in cerebral blood flow. Understandably, the research activity on cerebral hypoperfusion has recently been extremely high. The CA1 region of the hippocampus is particularly vulnerable to hypoxic conditions. The hippocampus, where cognitive processes are believed to depend on changes in synaptic efficiency, is a brain region extremely rich in glutamatergic synapses and sensitive to Glu excitotoxicity. It has recently been reported that glutamate-induced excitotoxicity and a cellular calcium overload are among the key factors of cell death in brain ischemia.

One promising neuroprotective intervention might involve modulation of the KYN pathway, which converts tryptophan into various compounds, e.g. KYN (an intermediate compound). KYN can be converted to KYNA, one of the few known endogenous broadspectrum antagonists of the excitatory amino acid receptors and especially the NMDA receptors. KYNA itself (or its derivatives) is neuroprotective under different pathological circumstances. KYNA is barely able to cross the BBB. On the other hand, KYN is transported across the BBB by a neutral amino acid carrier, and is converted to KYNA by kynurenine aminotransferase within the brain. KYN administration has proved to be neuroprotective in histological studies. However, the neuronal degeneration marker used, Fluoro Jade B, does not discriminate between apoptotic and necrotic cell damage. Moreover, recent studies have indicated that Fluoro Jade B can stain neurons degenerating as a result of an acute insult, and it can label activated microglia and astrocytes during a chronic neuronal degenerative process. All these points should be taken into consideration. The systemic administration of KYN together with PROB resulted in concentrations of KYNA in the brain which have proved to be neuroprotective in histological and behavioral studies. However, relatively little is known about its impact on the outcome of synaptic plasticity.

A novel finding here is that the administration of KYN (+PROB) once before and 4 times after 4VO induced transient global ischemia proved neuroprotective in our histological studies, and also reduced (nearly abolished) the impaired LTP induction in the Schaffer collateral-CA1 pathway in adult rats. Interestingly, the IO curves of the controls, and of ischemic +KYN+PROB-treated rats did not display significant changes. This suggests that it is the machinery of LTP that is injured rather than the basal functions of the pyramidal cells and synapses. It should additionally be taken into consideration that, though the FJ-B labeling indicates massive neuronal degeneration in the untreated 4VO group, the NeuN positivity

demonstrates that nearly 1/3 of the neurons in this group remained intact, in spite of the complete ischemia.

After transient global ischemia, the surviving neurons displayed normal transmission, except for the reduction in the maximum level of fEPSPs that seems to be a consequence of the cell number reduction due to ischemic cell death. The impaired LTP induction should reflect deficits in the machinery specific to LTP induction in the individual surviving neurons. Although the mechanism is not yet known, we have demonstrated for the first time here that treatment with KYN (and PROB) rescues the Schaffer collateral-CA1 synapses from impaired LTP induction after transient global ischemia.

In the second series of experiments, oxaloacetate was administered in the first 30 min of the reperfusion period and was found to prevent the LTP impairment caused by ischemia without affecting the basal glutamatergic synaptic functions, as it was concluded from the results of the paired-pulse facilitation and the IO curves. The most apparent explanation is that intravenous oxaloacetate causes an increased elimination of excess Glu from the hippocampus extracellular space by virtue of its blood Glu-scavenging properties at the higher oxaloacetate doses tested.

In the future, the administration of oxaloacetate to human might open up new therapeutic possibilities basically different from those involving the administration of Glu receptor antagonists: 1) In contrast which the use of Glu receptor antagonists, the activity of Glu scavengers in stimulating the brain-to blood Glu efflux is self-limiting, since this activity progressively diminishes as the elevated brain Glu level decreases to concentrations below the threshold of activation of the Glu transporters on the brain vasculature (i.e below their K_m values). 2) Blood Glu scavengers do not affect ionotropic Glu receptors, whereas Glu receptor antagonists obviously do, and hence they will not block the beneficial effects of Glu in the neurorepair proceeding after brain injury. Our experiments have provided the first evidence that the blood Glu scavenger oxaloacetate improves the impaired LTP in the hippocampal CA1 region after ischemia, and furnishes new insight into a novel mechanism for the treatment of ischemic stroke.

Scientific publications of the author

Original papers directly related to the thesis

European Journal of Pharmacology 604 (2009) 51-57. Impact factor: 2,376

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

Máté Marosi; János Fuzik; Dávid Nagy; Gabriella Rákos; Zsolt Kis; József Toldi; Vivian I Teichberg; Angela Ruban-Matuzani; Tamás Farkas

Neurobiology of Disease 32, (2008) 302-308. Impact factor: 4,377

Kynurenine diminishes the ischemia-induced histological and electrophysiological deficits in the rat hippocampus

Sas, K., Robotka, H., Rozsa, E., Agoston, M., Szénási, G., Gigler, G., **Marosi, M.**, Kis, Z., Farkas, T., Vecsei, L., Toldi, J.

Papers connected to the thesis

Cellular and molecular neurobiology (2009) 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

Nagy D, **Marosi M**, Kis Z, Farkas T, Rakos G, Vecsei L, Teichberg VI, Toldi J.

Journal of Neuroscience Methods 156 (2006) 231–235 Impact factor: 1,894

Hippocampal (CA1) activities in Wistar rats from different vendors. Fundamental differences in acute ischemia

Marosi M, Rakod G, Robotka H, Nemeth H, Sas K, Kis Zs, Farkas T, Lür Gy, Vecsei L, Toldi J

Other papers

Neuropharmacology 47 (2004) 916–925 Impact factor: 3.734

Kynurenine administered together with probenecid markedly inhibits pentylenetetrazol-induced seizures. An electrophysiological and behavioural study

Nemeth H, Robotka H, Kis Z, Rozsa E, Janaky T, Somlai C, **Marosi M**, Farkas T, Toldi J, Vecsei L.

Cummulative impact factor: 14,873