

PH.D. THESIS

**STUDIES ON NEUROPROTECTION
IN TRAUMA AND STROKE MODELS**

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Introduction

There may be an appreciable number of harmful effects in the background of disorders of the nervous system, and neurological diseases are therefore very diverse. Depending on their origin, they can be divided into traumatic, vascular (e.g. stroke), inflammatory (e.g. encephalitis), autoimmune (e.g. multiple sclerosis), degenerative (e.g. Alzheimer's disease) and tumorous (e.g. glioma) neurological disorders.

A few decades ago it was a general mindset that severe damage to the nervous system (without reference to the origin) could cause irreversible functional or structural changes. Clinical experience has shown that the symptoms and simultaneously the condition of patients with brain damage worsen in the course of time. The results of research in recent years have provided the explanation of the progression. It has become known that, in cases of traumatic brain injuries or stroke, prevention of the secondary damage that follows the primary damage can considerably reduce the death rate and the development of permanent lesions. This recognition gave a new line to the therapy principles and to the protocols applied.

Our research has focussed on the underlying and accompanying pathophysiologic factors of traumatic brain injuries and ischaemic brain damage. In the course of our studies, we adopted widely accepted trauma and stroke models.

It is well known that stroke is the most frequent acute disorder among neurological diseases and is the third leading cause of death. Traumatic brain damage is the most common cause of permanent brain damage, besides vascular catastrophes of the brain. In the developed, highly motorized countries, the number of traumatic brain injuries increase from year to year. Nearly half of accidental deaths are connected with cranial or brain injuries.

There are common components in the pathophysiological events that play a role in the brain tissue damage of different origins. In particular, the majority of central nervous system conditions or injuries lead directly or indirectly to a transient or permanent blood supply deficiency. Through glucose and oxygen deficiency, a deficient blood supply induces a multistage cascade-like process that results in an energy crisis for the nerve tissue. A total energetic catastrophe is typical of the central part of the damaged area (core region) causing rapid nerve cell death (necrosis). In the neighbouring region (penumbra region), where the energy crisis is less explicit, the cells mainly die through apoptosis, the process of delayed neuronal death. As apoptosis is a

delayed and long-lasting process, it is possible to use cell death-preventing methods. These features of the penumbra have become the main target of neuroprotective strategies.

Increased glutamate (Glu) release plays an important role in the complex pathophysiological progresses leading to nerve cell death. Excitotoxicity, caused by increased Glu release, plays an important role in the rise of nerve cell death to the tissue level. In excitotoxicity prevention, an important role is attributed to the Glu transporters of the axon terminals of the neurones and of the glia cell membranes that are capable of binding and taking up the Glu released during the nerve function. As a result of their function, the Glu level returns to its normal value. Na⁺-dependent Glu transporters can also be found in the endothelial cells of the brain vessels, which allow the Glu to pass through the blood-brain barrier. In this way, the blood can indirectly control the extracellular concentration of Glu. However the Glu efflux from the brain to the blood is limited as the average concentration of Glu in the plasma is 40-60 μM, i.e. a multiple of the concentration in the cerebrospinal and the interstitial fluids (1-10 μM). This concentration difference does not favour the Glu flux from the brain to the blood. However, the high extracellular Glu concentration that accompanies nervous system injuries enables the endothelial cells to take up and accumulate Glu. If the intracellular Glu concentration is higher than the plasma concentration, Glu is transported to the blood through the luminal membrane of the endothelial cells via facilitative transport. Decreasing the plasma Glu concentration can increase the Glu efflux from the brain to the blood. Gottlieb et al. used two resident enzymes of the blood to decrease the Glu concentration: glutamate-pyruvate transaminase (GPT) and the glutamate-oxaloacetate transaminase (GOT), which transform Glu into 2-α-ketoglutarate and aspartate in the presence of the co-substrates of Glu (pyruvate (Pyr) and oxaloacetate (OxAc)). The transformation of Glu into 2-α-ketoglutarate and aspartate is a bidirectional process. When OxAc or Pyr is used in high concentration, the process can be offset to 2-α-ketoglutarate and aspartate formation, resulting in a decrease in the Glu concentration.

This points to the fact that the elevated extracellular Glu level of the brain and the consequential excitotoxicity can partially be controlled via change of the blood Glu level.

Aims

Injury model

Evans Blue (EB) and TTC labelling in a cold lesion model

In our experiments we used the rat cortex to investigate the mechanism of cellular injury caused by a cold lesion.

1) Our first aim was to adapt a histological method, which is suitable for the short-term detection of the lesioned brain area, the extent of the perilesional rim and the damaged cells.

Investigation of the effects of pre- and post-traumatic DHEAS treatment

In a focal cortical cold lesion model we evaluated the effects of both pre- and post-traumatic treatment with dehydroepiandrosterone sulphate (DHEAS), a compound that has proved to be neuroprotective in other models. In our experiments, we investigated the efficacy of pre- and post-traumatic DHEAS treatment in the prevention of injury-caused tissue damage. In the same experimental model, we investigated the effects of pre- and post-traumatic 17 β -oestradiol (E₂) treatment, and to what extent the DHEAS effects are influenced in the presence of letrozole, an aromatase inhibitor.

2) Our next aim was to investigate the neuroprotective ability of the examined steroids in the induction a focal cortical cold lesion injury.

Stroke model

Investigation of the neuroprotective effects of the Glu scavenger OxAc

3) In these experiments, we induced tissue damage by artificial thrombosis in the rat cortex, and investigated whether OxAc (as a potentially neuroprotective substance) influences the extent of the lesion and the number of the degenerated neurones.

Methods

Experimental animals

In our experiments, we used male Wistar rats, weighing 300-350 g. The animals were anaesthetized by the intraperitoneal (ip.) administration of a mixture of ketamine and xylazine. During the experiments supplementary doses were administered in order to maintain proper anaesthesia. The handling and laboratory use of the experimental animals fully satisfied the EU-harmonized standards and received the approval of the University Ethical Committee.

Injury model

In one group of animals, a cold lesion was induced in the primary motor cortex by applying a cooled copper cylinder. The copper cylinder (diameter 2 mm), which was specially designed for these experiments, was cooled with a mixture of acetone and dry ice in a two-phase system (-78 C). The cooled thermod was placed onto the surface of the exposed cortex for 30 sec. During surgery, the body temperature of the animals was maintained at 37 ± 0.5 °C with an infrared lamp. Sham-operated animals served as controls. The procedure in the sham-operated animals was the same as described above, except that the copper cylinder thermod otherwise used to evoke the cold injury was at room temperature. After the surgical procedures, different staining procedures were applied for lesion detection.

TTC reaction

TTC (2,3,5-triphenyltetrazolium chloride) a commonly used indicator of neuronal death can also be used for the rapid and simple detection of the efficacy neuroprotective agents. Four hours after the induction of the lesion, the animals were killed by decapitation and their brains were removed from the skull. Thereafter, 0.4-mm-thick coronal slices were cut throughout the ischaemic region with a vibratome. The slices were placed in a 1% TTC solution dissolved in artificial cerebrospinal fluid. When the reaction was complete, the slices were fixed with 4% paraformaldehyde solution and subsequently left to stand overnight. The TTC reaction was also used in the case of the sham-operated animals.

EB staining

EB staining is a method frequently used to monitor the dysfunctions of the blood-brain barrier. EB injected into the circulating blood is bound to serum albumin. Where the blood-brain barrier is damaged, the stain bound to albumin passes into the brain tissue and is taken up by the damaged cells.

We applied EB labelling in 13 cases of cold-lesioned animals. 2% EB solution was administered through the tail vein of the animals. After 4 hours of survival, the animals were perfused transcardially. The brains were removed and post-fixed overnight. Coronal sections (50 μ m) encompassing the EB-labelled area were cut with a vibratome. The sections were observed under a fluorescence microscope at an excitation wavelength of 530-550 nm and an emission wavelength of 590 nm. EB labelling was similarly applied in the sham-operated animals. In this case, as a separate control group, we used animals that had received the same volume of saline instead of the EB stain.

DHEAS and E₂ treatment

In our study, we investigated the effects of DHEAS treatment on the extent of the cold lesion-induced tissue injury. Six different groups were examined. DHEAS was applied as pre-treatment in one group and as post-treatment (50 mg/kg) in another group of cold-lesioned animals. Similarly, E₂ treatment (35 mg/kg) was administered in two further groups of experimental rats. The control animals received distilled water. In the sixth group, an aromatase inhibitor, letrozole, was administered after DHEAS pre-treatment. One hour after lesion induction, the experimental animals were killed by decapitation. Thereafter, 0.4-mm-thick coronal slices were cut throughout the damaged region with a vibratome. The injury was visualized by using a 1% solution of TTC. The surface area of the injured regions on the stained sections was measured and the total injured brain volume was determined in cubic millimetres.

Statistical analysis

For further statistical analysis of the data, Origin (version 7.0; OriginLab Corp., Northampton, MA) and SPSS12 (Chicago, IL) softwares were applied. The measured parameters were given as means \pm S.E.M. To compare the groups, one-way ANOVA followed by the *post hoc* Bonferroni test was applied. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ values were considered significant.

Stroke model

Photochemically initiated thrombosis, the photothrombotic lesion is a widely used stroke model. During this method, a photosensitive stain, Rose Bengal, is injected into the blood circulation intravenously. Under light exposure, the stain induces platelet aggregation and thrombus formation. After tail-vein injection of Rose Bengal (30 mg/kg), the somatosensory cortex was illuminated for 20 minutes through the profoundly cleaned skull with a cold light fiberoptic tool 5 mm in diameter. In one group, the animals received OxAc solution after the light exposure. OxAc solution (1.2 mg/100 g, 50 μ mol/minute) was administered through the tail vein too, during a 30-minute period. Both the control and the OxAc-treated groups were perfused transcardially after 4 hours of survival. The brains were removed and post-fixed in a 4% solution of paraformaldehyde. Thereafter, 36- μ m-thick slices were cut throughout the damaged region with a microtome. The injured areas caused by the vascular occlusions and the degenerated neurones were visualized by Fluoro-Jade B® (FJB) staining.

FJB staining

FJB is an anionic fluorochrome, which is able to stain degenerated neurones selectively. Its advantages are its simplicity and sensitivity, and it can also be combined with other immunohistochemical and fluorescent procedures.

First, decreasing ethanol series were used: We placed the slices into 96% ethanol for 3 minutes, then 70% ethanol for 2 minutes and finally into distilled water for 1 minute. After this, the slices were transferred to 0.06% KMnO₄ solution for 15 minutes. The sections were next rinsed in distilled water, and then placed in a 0.001% FJB staining solution for 30 minutes. The staining procedure ended with rinsing with three changes of distilled water for 1 minute per change. The sections were air-dried overnight and then cover-slipped with Fluoromount®. The sections were subsequently analysed with a fluorescence microscope at an excitation wavelength of 470-490 nm and an emission wavelength of 520 nm.

Statistical analysis

For the statistical analysis, SPSS for Windows version 9.0 software was used. The parameters are given as means \pm S.D. For comparison of the mean values of the control and the treated groups, the unpaired-sample t-test was applied. A * p value of < 0.05 was considered significant.

Results

In the first part of our experiments, we used a cold lesion model to study the brain injuries. The temporal and spatial changes of the cold-damaged area were monitored with TTC and EB staining. EB staining has proved to be a useful method: through its fluorescent features, the area of the brain flooded with EB due to a blood-brain barrier disruption can easily be visualized. In the affected brain area (penumbra) the damaged neurones also take up EB, so the EB-indicated pyramidal cells become visible in a short time. They cannot be visualized in such a detailed way with TTC staining, but this also proved to be an appropriate test. As concerns our first aim, we could state that EB and TTC staining are suitable for measurements on the neurodegeneration and neuroprotective processes. This was proved in our experiments in which we examined the effects of steroids in cold lesion models. Our aim was to establish whether DHEAS and E_2 treatment could reduce the extent of the damaged area and whether they are effective in pre- and post-treatments. As concerns our second aim, we found that DHEAS and E_2 are effective in pre-treatment and also as post-treatment, and significantly reduce the extent of the lesion. We could block the protective effect of DHEAS with letrozole. Letrozole is an aromatase inhibitor that blocks the aromatic transformation of DHEAS into E_2 . We assume that the positive effect DHEAS on cell death is transmitted by E_2 .

In a series of experiments, we tested a completely new aspect of neuroprotection: we examined the effects of OxAc in a stroke model. The stroke was modelled with a photothrombic lesion. The vessel occlusion caused cortical damage which was visualized with FJB staining. As FJB indicates the degenerated neurones with high specificity, its use allows examinations not only of the extent of the lesion, but also of the number of degenerated neurones per unit area can be examined. In our experiments, we examined the manner in which OxAc, a possible neuroprotective agent, influences the size of the lesion and the number of damaged cells. We demonstrated that OxAc has a neuroprotective effect, manifested in decreases in the extent of the lesion and in the number of degenerated neurones. A further important observation was that the animals that received OxAc treatment after the lesion had a greater survival rates.

Treatment with Glu scavenger OxAc is a novel approach among the currently researched and applied neuroprotective strategies. As regards our third aim, we established that OxAc administration (ip.) proved to be an effective neuroprotective intervention. Its possible

therapeutic use demands further electrophysiological, histological, biochemical and toxicological examinations.

Conclusions

During our experiments we succeeded in answering the questions raised, and in achieving our objectives:

Trauma model

1) The EB staining used in our experiments proved appropriate for examination of the cold lesion-induced tissue damage and the penumbra region. With this method (as compared to other immunohistochemical processes), the extent of the lesion and the damaged cells can be visualized in a shorter time. We are the first to detect EB-stained pyramidal cells in the penumbra region. Further advantages of the method are its technical simplicity, sensitivity, great reproductivity and cost-effectiveness.

2) DHEAS and E₂ proved effective as both pre-treatment and post-treatment in the cold lesion model. Both treatment forms significantly reduced the extent of the tissue lesion. Letrozole treatment abolished the neuroprotective effect of DHEAS, which may reflect the fact that the protective effect of DHEAS is transmitted by E₂.

Stroke model

3) We are the first to examine the effect of OxAc in an ischaemic model through histological methods. We demonstrated that OxAc has a neuroprotective effect manifested in decreases in the extent of the lesion and in the number of degenerated neurones.

Original papers directly related to the thesis

Nagy D, Marosi M, Kis Z, Farkas T, **Rákos G**, Vécsei L, Teichberg VI, Toldi J

Oxaloacetate decreases the infarct size and attenuates the reduction in evoked responses after photothrombotic focal ischaemia in the rat cortex

Cellular and molecular neurobiology 2009 Sep;29(6-7):827-35

(Impact factor:2.107)

Rákos G, Kis Z, Nagy D, Lür G, Farkas T, Hortobágyi T, Vécsei L, Toldi J

Evans Blue fluorescence permits the rapid visualization of non-intact cells in the perilesional rim of cold-injured rat brain

Acta Neurobiologiae Experimentalis 2007;67(2):149-54

(Impact factor:1.337)

Juhász-Vedres G, Rózsa E, **Rákos G**, Dobszay MB, Kis Z, Wölflin J, Toldi J, Párducz A, Farkas T

Dehydroepiandrosterone sulfate is neuroprotective when administered either before or after injury in a focal cortical cold lesion model

Endocrinology 2006 Feb, 147(2):683-6

(Impact factor:4.752)

Papers connected to the thesis

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

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

European Journal of Pharmacology 2009 Feb 14;604(1-3):51-7

(Impact factor:2.585)

Lür G, **Rákos G**, Juhász-Vedres G, Farkas T, Kis Z, Toldi J

Effects of dehydroepiandrosterone sulfate on the evoked cortical activity of controls and of brain-injured rats

Cellular and molecular neurobiology 2006 Oct-Nov;26(7-8):1505-19

(Impact factor:2.107)

Other papers

Marosi M, **Rákos G**, Robotka H, Németh H, Sas K, Kis Z, Farkas T, Lür G, Vécsei L, Toldi J

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

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(Impact factor:2.295)

Kis Z, **Rákos G**, Farkas T, Horváth S, Toldi J

Facial nerve injury induces facilitation of responses in both trigeminal and facial nuclei of rat

Neuroscience Letters 2004 Apr 1; 358(3):223-5

(Impact factor:1.925)