Histopathological and therapeutical experiments in severe experimental head injury models

PhD thesis

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“Discovery consists of seeing what everybody has seen and thinking what nobody has thought”

*Albert Szent-Gy**ö**rgyi*

I. INTRODUCTION, LITERATURE BACKGROUND

Epidemiology of head injury

Traumatic brain injury (TBI) is the leading cause of mortality in the active/working population and a major cause of disability in people under the age of 35. While the mortality rate of head injuries is 20-25% in western societies, this figure in Hungary is 45%. In Europe 1.600.000 head injured patients receive hospital care, producing a brain injury rate of 235/100.000/year. The leading causes of TBI are traffic accidents, falls, violence and sport related injuries. The most imperiled demographic groups are males and individuals living in regions characterized by socioeconomic deprivation.

The average cost per inpatient with severe traumatic injury is around 6,000 Euro in the most developed European countries. The costs due to initial hospitalization are only a small part of total costs. A total lifetime costs per TBI case in the US is around $200.000. According to some estimates TBI will become the third most common cause of death globally by the year 2020.

Classification of head injuries

The most commonly used method of determininge injury severity is the Glasgow Coma Scale (GCS) developed by Teasdale et al., which distinguishes mild (GCS=13-15), moderate (GCS=9-12), and severe (GCS=3-8) injuries. According to the traditional classification of head injuries we can discern open and closed injuries. Patomechanisms of injury are differentiated as primary or secondary changes. Primary injuries are the result of mechanical forces that producetissue damage at the moment of injury. The damage may affect blood vessels, axons, neurons, and glia in a focal or diffuse pattern.

Diffuse axonal injury

Diffuse axonal injury (DAI) is the sum of all axonal changes caused by traumatic brain injury, which are scattered throughout the brain in the midst of normal axons in a widely distributed in mostly normal parenchyma.

DAI has two distinct morphological appearances: axonal swelling/axonal bulb formation (AS/B) and ultrastructural (neurofilament) compaction (NFC). There are many different animal models for DAI, however, none of these are able to represent 100% of the overall dimension and time course of human DAI. Therefore in animal models axonal injuries are called traumatic axonal injury (TAI), while DAI is used for human cases.

Axonal swelling/axonal bulb formation (AS/B)

The phenomenon of diffuse axonal injury was first described by Strich et al. After *post mortem* examination of the white matter of severely head injured patients, balloon-like axonal dilations were observed in a scattered pattern along intact axons, while wallerian and myelin degratation was observed on the distal segment. The currently used name for DAI comes from Adams. Since Strich, Adams and his colleagues emphasized the first time, that axonal injury is a primary consequence of head injury, and it does not develop as a consequence of secondary injury. According to Povlishock et al., these axonal damages are induced by shearing forces caused by moderate or severe traumatic brain injury. They found that DAI

process that is gradually ongoing in time in the majority of the damaged axons. During this process, focal axolemmal permeability disruption occurs almost immediately (<5 minutes) post-injury along in the injured axonal segments.

Focal damage of the axolemma is accompanied by morphological characteristics, such as the swelling of mitochondria, disappearance of neurotubules, neurofilament modifications and impaired anterograde axonal transport. This results in the accumulation of carried cell components and other substances, that manifest in axonal swelling. Over time, the axonal swelling increases, resulting in the detachment of the affected axonal segment in a ballon form, creating proximal axonal bulbs. The distal axonal segment undergoes wallerian degeneration.

The time lapsed from the beginning of axonal alterations until the formation of axonbulbs and axonal disconnection depends on the severity and type of trauma, and on the species. In humans, this time is measured in hours and days, thus the available therapeutic time frameis much longer than in experimental animals.

Ultrastructural (neurofilament) compaction (NFC)

Ultrastructural compaction was discovered by Povlishock et al. with electronmicroscopic analysis of the brainstem in immediate (5 minutes) post injury perfused animals. They observed that the distance between neurofilament sidearms was almost halved, and the number of microtubules and neurofilament was greatly reduced. Based on their theory, the mechanism of NFC is the following: as a consequence of head injury, small sized pores are opening on a short section of the affected axon (mechanoporation), the regulatory role of axolemma is terminated and calcium ions enter into the axon. The greatly increased concentration of calcium results in calpain activation, which cleaves non-erythroid alpha-II spectrin to 145 and 150 kD sized subunits (calpain-mediated spectrin proteolysis, CMSP). Spectrin is a constituent of the subaxolemmal cytosceletal network, and it can also be found around the mitochondria as well. In the early phase (15-30 minutes) the cleavage of spectrin is localized to the subaxolemmal and perimitochondrial area, then it shifts to the cytosceletal network of the affected axon, and its degradation causes further permeability disturbance, and the activation of intra-axonal proteolytic cascades, which in turn leads to irreversible axonal damage.

For the histological detection of NFC, RMO-14 antibody is used against of the medium-sized neurofilament subunit (NF-M). After the dephosphorilation of the neurofilament's sidearm, RMO-14 antibody is able to bind to the "rod domain" of NF-M. Earlier observations assumed that NFC and AS/B occur in the same damaged axons. Today, however, it has become clear that in most cases, these two morphological changes occur in different axon populations and rarely within the same axon.

Experimental therapeutic influence of diffuse brain injury

Pituitary adenylate-cyclase activating polypeptide (PACAP)

Both necrotic and apoptotic enzyme cascades play a role in DAI, therefore processes that are likely to inhibit both, deserve special consideration. This dual targeting is especially important, since according to some researchers, the inhibition of apoptotic processes cannot save neurons after mitochondrial damage, such as the dissociation of electron-transport chain, and cytochrome c release. Instead of apoptotic cell death, it causes only the activation of necrotic cascade, therefore, and evokes a shift between the two enzymatic processes.

PACAP is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon family and was first isolated from the pituitary gland.

In vitro studies demonstrated PACAP's anti-apoptotic and anti-inflammatory effects. *In vivo* studies indicated that PACAP crosses the blood-brain barrier (BBB) and is equally effective before and after administration in rats' experimentally induced global and focal cerebral ischemia, as well as in retinal degeneration.

In the last few years, our research group examined the neuroprotective effect of PACAP in a model of diffuse experimental TBI. The effective dose in earlier studies with cerebral ischemia (125 μg administered intravenously (i.v.) prior to injury), did not exert a beneficial effect in TAI, however, intracerebroventricular (i.c.v.) injection proved to be effective. The treatment dose in our experiment was based on the results of the above mentioned research group, that treatment with an i.c.v. injection of 100 μg PACAP was able to significantly reduce the density of impaired axons in the corticospinal tract (CSpT).

Poly(ADP-ribose)polymerase (PARP)-inhibition

PARP was known as a DNA repair enzyme. It is activated by DNA breakage caused by oxidative stress, resulting in the transfer of ADP-ribose units from NAD^+ to nuclear proteins. The overactivation of this highly energy demanding process, induced by cumulative stress, results in the depletion of NAD^+, loss of ATP, and through the collapse of the energy homeostasis of the cell, it may result in cell death.

While long-term inhibition of PARP may induce carcinogenesis and mutagenesis, it may contribute to the normalization and maintenance of the energy homeostasis in the acute stage.

Komjáti et al. have recently (2005) found, that PARP inhibition not only inhibits free radical-induced necrosis, it plays a direct a role in initiating apoptotic processes via apoptosis-inducing factor (AIF). Furthermore PARP modulates the release of inflammatory mediators and cytokines via NF-kappaB pathway.

In recent years, several PARP inhibitors have been tested in various models of TAI, including 3-aminobenzamide, which significantly reduced the extension of lesion size in cold-induced brain injury-model. Interestingly, similarly to the neuroprotective effect of calpain inhibition, the pathobiological of the effect of PARP-inhibition has not been revealed yet.

In our experiments, we examined the effect of the newly developed PARP-inhibitor, L-2286 (2-[(2-piperidin-1-yletil)thio]quinazolin-4(3*H*)-one) in Marmarou's impact acceleration head injury model.

Diffuse neuronal injury (formation of "dark" neurons)

More than a century ago it was discovered that the brains of those patients who died of various neurological diseases, contained neurons whose nucleus, cytoplasm and main dendrites were dramatically shrunken. These neurons had increased affinity to bind basic neurohistological dyes. Traditionally these cells were called "dark" neurons. Since the early 80's the presence of "dark" neurons was described in a numerous animal models of different neurological diseases (hypoglycemia, hyperglycemia, status epilepticus, focal ischemia and four-vessel ischemia).

In the early 90's, Gallyas et al. observed that "dark" neurons and axons can be produced by both *in vivo*, and *post mortem* mechanical injury. According to electronmicroscopic observations, the ultrastructural elements are intact, but were dramatically compacted (ultrastructural compaction) in all freshly produced "dark" neurons. Those cells had an increased electrondensity, their endoplasmic reticulum (ER) cisternae were contracted, and Golgi cisternae were dilated, while the mitochondria and tother multivesicular bodies remained unchanged in volume. A characteristic of "dark" neurons is, that if the soma of a neuron is affected, its dendritic tree is also effected, and *vice versa.*

This phenomenon can be explained the compaction starts at one single point on each soma-dendrite domain, and then it spreads to the whole soma-dendrite domain. Gallyas et al. (1992) explained the mechanism of this progression by the cytoplasm of neurons (and other cells) containing a microtrabecular gel-structure, which is anchored to ultrastructural elements, that stores free energy in non-covalent bindings. After the initialization of this gel-structure at a single point, it is capable of propagating a phase transition (gel-to-gel phase transition) through the impact of stored energy. Those gels have two or more metastabile stages. Each metastable stage with different energy levels belongs to a different polymer-molecule conformation, different water content and different volume. After transmission of sufficient activation energy to the higher energy level stage, its one single point switches over to a stage of lower energy value, but energetically more stable level. This released energy serves as activation energy, and initiates chain-reaction on the neighboring points (domino-principle). In the case of "dark" neurons, the phase transition can be initialized by pathometabolic processes (ischemia, hypoglycemia, status epilepticus or mechanic impact). Contraction of gel-structure pulls to itself other anchored ultrastructural elements and squeezes out fluid from the soma-dendrite domain along with the affected axon-sections.

II. AIMS

I intended to investigate new experimental therapy opportunities that influence diffuse axonal injury, and aimed to explore the further details of the formation of ultrastructural compaction. During my work the following questions were examined:

1., Does the PACAP have a neuroprotective effect in the rat head injury model of central fluid percussion (CFP)?

2., Does the newly developed L-2286 PARP inhibitor have a neuroprotective effect in the rat head injury model of Marmarou's impact acceleration?

3., What is the fate of (ultrastructural compacted) "dark" neurons produced by transient focal cerebral ischemia in a non-necrotic and non-excitotoxic environment?

1. THE EFFECT OF PACAP TREATMENT IN THE CENTRAL FLUID PERCUSSION HEAD INJURY MODEL

1.1. MATERIALS AND METHODS

Animals

Male Wistar rats weighing 350-380 g were housed in cages with free access to food and water in the animal facility of the Department of Neurosurgery in Pécs.

Rat central fluid percussion injury model

Animals were pre-anesthetized in a bell jar for 5-minutes with a mixture of 4% isoflurane, 70% N_2O and 30% O_2. After endotracheally intubating the animals, anesthesia was maintained with a mixture of 1-2% isoflurane in 70% N_2O and 30% O_2. Thereafter animals were placed in a stereotactic device and a midline incision was made to expose the skull from the bregma to the lambda. Two 1-mm holes were drilled in the dried right frontal and occipital bones 1 mm from the bregma and the lambda, for the insertion of fixation screws to secure the "crown". A 4.8 mm-diameter craniotomy was then made, centered in the midline between the bregma and the lambda above the intact dura. A plastic injury cap was then affixed over the craniotomy using silicone adhesive and cyanoacrylate. As a main part of the "crown" a plastic injury cap was affixed over the craniotomy. Dental acrylate was applied around the cap and the two earlier fixated screws, to make the "crown" at same height level as the cap. When the dental cement was completely hardened, the injury cap was filled with 0.9% saline. Anesthesia was then discontinued and animals were attached to the fluid percussion device and were subjected to an injury at a magnitude of 2 atm, which is considered to be of moderate severity. Sham-injured controls were prepared for injury in the same manner but were not injured. After the injury, rats were monitored for the recovery of spontaneous respiration, if spontaneous respiration was not evident after 20 seconds, animals were rapidly reintubated and ventilated with 100% O_2 until spontaneous respiration was regained.

I.c.v. PACAP treatment

A single bolus of 100 µg PACAP was dissolved in 5 µl physiological saline and administered i.c.v. 30 min after injury in to the right ventricle (AP:-1.0; L: 1.5; V:-3.5 from the bregma). The vehicle-treated group received the same volume of physiological saline in the same way.

Immunohistochemistry

Two hours after injury rats were sacrificed with an intraperitoneal (i.p.) injection of sodium pentobarbital, and were transcardially perfused with 4% paraformaldehyde. Brains along with the brainstem were removed and immersed in 4% paraformaldehyde overnight. A medial, 5-mm-wide block of the brain was excised using a sagittal brain blocking device, and 30 µm vibratome sections were made. Until the start of the immunohistochemical procedure, slides were stored in 0.1 M phosphate buffered saline.

Two immunohistochemical protocols were used. For the detection of impaired axoplasmic transport we used a polyclonal antiserum targeting the C-terminus of betha-

amyloid precursor protein (β-APP). For the detection of NFC, an RMO-14 antibody was used, which is able to bind to the rod domains of altered neurofilament NF-M subunits.

Histological analysis with Image-Pro Plus v5.0.1

APP or RMO-14 immunopositive (IP) axonal profiles were examined using a light microscope interfaced with a digital camera and a computer-assisted image analysis system (Image Pro Plus v5.0.1). According to previous descriptions of the main foci affected by DAI the area of the CSpT and the medial longitudinal fascicle (MLF) of the brainstem were analyzed.

Statistical analysis

Differences between the densities of IP axonal profiles (expressed as mean number/mm^2) in PACAP and vehicle-treated rats ware compared with Student *t*-test. A difference was considered significant when p value was less than 0.05.

1.2. RESULTS

After light microscopy and statistical analysis we concluded that treatment with 100 μg PACAP 30 min after the induction of moderate CFP injury resulted in a significant reduction in the density of APP- and RMO-14 IP axonal profiles in the CSpT, compared to vehicle-treated animals. In the MLF, no significant difference was observed between the densities of APP- or RMO-14 IP axons in PACAP versus vehicle-treated animals (**Table 1.**).

APP IP axons	***Vehicle***	***PACAP***	**RMO-14 IP axons**	***Vehicle***	***PACAP***
CSpT	560.96±64.36	290.04±36.93 (p<0.05)	*CSpT*	283.31±29.91	155.71±31.52 (p<0.05)
MLF	307±24.17	265.57±10.81	*MLF*	272.31±20.1	265.7±33.46

Table 1. Mean density of APP and RMO-14 IP axons in the CSpT and in MLF in PACAP versus vehicle-treated animals. Data expressed as mean number/mm^2 ± SEM. p <0.05 versus vehicle-treated rats

1.3. DISCUSSION AND CONCLUSION

Our work is the first study which demonstrated in the CFP model the neuroprotective effect of PACAP in TAI. Our results confirmed previous findings from the impact acceleration model of diffuse TAI, indicating that PACAP is an efficient inhibitor of impaired axoplasmic transport and NFC associated with axonal injury. Furthermore, the results were consistent with previous investigations demonstrating the neuroprotective effects of PACAP *in vitro* conditions and *in vivo* observations. *In vitro*, PACAP effectively inhibited apoptosis in various cell lines. *In vivo*, PACAP reduced the infarct size in models of both transient and permanent focal cerebral ischemia. In global cerebral ischemia i.c.v. or i.v. PACAP treatment prevented ischemic neuronal death in the CA1 region of the hippocampus. In models of Parkinson's disease it protects the dopaminergic nigrostriatal neurons from apoptotic cell

death. PACAP significantly inhibited the activation of caspases and other key molecules in the apoptotic cascade, playing important roles in the pathogenesis of TBI. A change in intraaxonal mitochondrial integrity plays an important role in the outcome of TBI. A pathological accumulation of calcium in the mitochondria inactivates the function of aconitase enzyme, a key mitochondrial enzyme that influences the viability of neurons. PACAP is capable of influencing this altered mitochondrial integrity through the inhibition of aconitase inactivation.

The key finding of this study is that i.c.v. administration of 100 μg PACAP 30 min after moderate CFP injury significantly reduced the density of both APP and RMO-14 IP axons. Eventhough impaired axoplasmic transport is not the only process that leads to axonal death, the prevention of such impairment and the discontinuity of anterograde transport should be considered one of the cornerstones in the therapy of diffuse TBI. It is important to note that the neuroprotective effect of PACAP was only proven significant in the CSpT. In this tract the density of damaged axons as well as their diameter and course are different from those analyzed in the MLF, this may partly account for the differences in our results. In the last few decades, despite the extensive research and testing for potential novel candidates capable of inhibiting the pathobiological mechanisms associated with the formation of DAI, none of the substances tested in clinical trials were proven to be effective.

In summary our experiments, we demonstrated that PACAP is capable of exerting its axono-protective effect not only in impact acceleration, but also in an animal model of CFP diffuse axonal injury. Based on previous and present findings, PACAP is considered a potential candidate for clinical trials in TBI.

2. L-2286: THE EFFECTS OF A NEW PARP INHIBITOR IN A RAT IMPACT ACCELERATION HEAD INJURY MODEL

2.1. MATERIALS AND METHODS

Animals

Male Wistar rats weighing 300-350 g were housed in cages with free access to food and water in the animal facility of the Department of Experimental Zoology and Neurobiology, University of Pécs.

Marmarou's rat impact-acceleration head injury model

All rats were first anesthetized in a bell jar for 5 min with 4% isoflurane in 70% N_2O and 30% O_2. After endotracheal intubation, rats were ventilated with 1.5% isoflurane in 70% N_2O and 30% O_2. A midline incision was made to expose the skull from the bregma to the lambda sutures. A stainless steel disc (10 mm in diameter and 3 mm thickness) was fixed centrally between the lambda and the bregma sutures using cyanoacrylate. To create a severe head injury, a 450 g weight was dropped from 2 m onto the stainless disc fixed to the rat's skull. After impact, the metal disc was removed and the animals were monitored during the recovery of spontaneous respiration. If spontaneous respiration it was not evident after 20 seconds, animals were rapidly reintubated and ventilated with 100% O2 until spontaneous respiration was regained. Sham-injured animals were prepared for injury in the same way, but were not injured.

Establishment of dose-response curve for i.c.v. L-2286

Thirty minutes after injury, a single bolus of 10, 50 or 100 μg/rat of L-2286 dissolved in 5 μl of physiological saline was administered in to the right ventricle through a craniotomy (AP:-1.0; L: 1.5; V:-3.5 to bregma). The vehicle-treated group received the same volume of physiological saline using the same method. Two hours post-injury, animals underwent the procedure that was described in earlier ”Immunohistochemistry”, with the only difference, that only the density of damaged APP IP axons in CSpT was determined.

Experimental protocol to test the post-injury administration regime

Half of the animals were treated immediately, and the another half 30 minutes post-injury trough the above mentioned method with a single bolus of i.c.v. 100 μg/rat L-2286. According to the two treatment time-points, we treated the vehicle-treated group with 5μl/rat of physiological saline as the L-2286 treated rats were. Two hours post-injury, animals underwent the procedure that was described in ”Immunohistochemistry”.

Histological analysis with Image-Pro Plus v5.0.1

APP IP axonal profiles were examined using a light microscope interfaced with a digital camera and a computer-assisted image analysis system (Image Pro Plus v5.0.1).

Statistical analysis

Differences between densities of IP axonal profiles (mean number/mm^2) in L-2286- and vehicle-treated groups were compared with Student’s T-test, results were considered statistically significant when p value was less than 0.05.

Beam-balance test

One day before the injury animals were repeatedly trained until they were capable of balancing on a 1.5-cm-wide square wooden bridge - suspended 60 cm above a foam pillow- for three consecutive trials of 60 sec each. All animal groups were tested 1 hour and 1-7 days after injury and scores from each group were analyzed. The scoring scale was the following: 1: balances with steady posture; 2: grasps the side of the beam and/or has unsteady movements; 3: hugs the beam or slips without falling; 4: attempts to balance on the beam but falls off; 5: drops over the beam or hangs on the beam and falls off and 6: does not attempt to balance on the beam.

Open-field test

The open-field test was used to measure spontaneous locomotor activity. The open-field is a box with a 70 × 70 cm floor and a height of 50 cm. Eighth days post–injury, rats were brought into the experimental room where they remained in their transfer cages for 30 min (acclimatization), after which each subject was placed for 5 minutes into the center of the

open-field box. Crossing, rearing and grooming were recorded with a video camera during the 5-minute test.

Elevated plus-maze test

The elevated plus-maze was used to measure anxiogenic behavior. The maze consists of 4 intersecting arms: 2 arms are fully open (open arms), while the other 2 are surrounded with 3 walls (closed arms). All rats were placed in the center of the maze and the following parameters were recorded for 5 minutes: number of head dips into the open arms, entries with the first limbs into the open arms, and the time spent in closed or open arms.

Statistical analysis

Test-results of each rat in the different experimental groups were totaled. Results of the beam-balance test were analyzed by one-way ANOVA with non-parametric Dunn's post hoc tests. Results of the open-field and the elevated plus-maze were analyzed by one-way ANOVA with Dunnett post hoc tests. In both tests the differences between groups were considered significant when $p<0.05$ and $p<0.01$.

2.2. RESULTS

Dose-response curve of i.c.v. L-2286

Thirty minutes post-injury treatment with 10 μg/rat of L-2286 i.c.v. did not reduce the density of APP IP axons in the CSpT. Eventhough 50 μg/rat of L-2286 i.c.v. reduced the density of damaged profiles, but that difference compared to vehicle-treated rats was not statistically significant. Of the three examined doses, only the 100 μg/rat of L-2286 i.c.v. was able to reduce significantly the density of APP-IP axons in CSpT (**Table 2.**).

	Vehicle	***10 μg***	***50 μg***	***100 μg***
Density	345.61±41.56	348.09±13.08	255.58±10.72	87.61±8.86 ($p<0.01$)

Table 2. The effect of i.c.v. L-2286 administered in different doses 30 min after severe impact acceleration head injury on the density of APP IP axons in the CSpT. Data expressed as mean number/mm^2 ± SEM ($p < 0.01$ compared to vehicle-treated group).

Therapeutic window

In the case of both APP and RMO-14 IP axons, 100 μg/rat L-2286 administered i.c.v. immediately, or 30 min post-injury; both treatments have reduced the density of impaired axons in similar degrees both in CSpT and in MLF, compared to vehicle-treated animals. However, in the case of RMO-14 IP axons, immediate post-injury treatment seemed to be

more effective, since a significant difference was observed in the density of damaged axons in both CSpT and MLF compared to treatment 30 min post injury (**Table 3.**).

APP IP axons	***Vehicle***	***L-2286 immediately post-injury***	***L-2286 30min post-injury***
CSpT	345.61±41.56	72.50±8.54 (p<0.01)	87.61±8.86 (p<0.01)
MLF	285.33±48.90	45.59±4.09 (p<0.01)	71.17±7.23 (p<0.01)
RMO-14 IP axons			
CSpT	88.29±6.55	5.56±2.98 (p<0.01)	45.81±5.04 (p<0.01)
MLF	131.66±21.39	16.01±3.04 (p<0.01)	42.92±2.3 (p<0.01)

Table 3. Densities of APP and RMO-14 IP axons in CSpT and MLF in animals treated with 5 μl/rat of i.c.v. vehicle and 100 μg/rat of L-2286 i.c.v. immediately, and 30 min post-injury. Data expressed as mean number/mm^2 ± SEM ($p < 0.01$ compared to vehicle-treated group)

Effect of 100 μg/rat L-2286 i.c.v. in beam-balance test

Vehicle-treated animals had the worst motor performance of the four assessed groups. Until the last test day, the animals in this group had not reached the performance level of sham-injured animals. Immediate post-injury treatment with 100 μg/rat L-2286 i.c.v. significantly improved motor performance at one hour post injury, compared to vehicle-treated animals. The group treated 30 min post-injury reached the maximum possible score on the fourth day (score 1). The motor performance of this group was significantly improved from the second examined time-point (one day) to the last time-point (seventh day) compared to the vehicle-treated group.

Comparison of two treatments shows that both treatments improved the impaired motor functions in the balance-beam test, but treatment with 100 μg/rat L-2286 i.c.v 30 minutes post-injury was more effective than treatment immediately after trauma (**Table 4.**).

Effect of 100 μg /rat L-2286 i.c.v. in open-field test

Immediate post-injury treatment with L-2286 did not significantly improve any of the three examined parameters compared to vehicle-treated animals. However, L-2286 treatment 30 min post-injury significantly improved the number of grooming activities compared to the vehicle-treated group, without any effect on the another two parameters (crossing and rearing) (**Table 4.**).

Effect of i.c.v. 100 μg /rat L-2286 in elevated plus-maze test

Results of the elevated plus-maze test showed, that the anxiety level was significantly lower in both L-2286-treated groups compared to the vehicle-treated animals, since rats spent significantly more time in the open arms than in the closed arms. Interestingly, the group treated immediately post-injury with L-2286, not only spent significantly more time in the

open arm than the vehicle-treated group, but also the sham-injured group. This finding was not observed in rats treated 30 min post-injury (**Table 4.**).

Beam-balance	*Sham injured*	*Vehicle*	*L-2286 immediately post-injury*	*L-2286 30 min post-injury*
1h (score)	1	6	3.8±0.58 p<0.05	4.8±0.2
1 day (score)	1	5.6±0.24	3.4±0.4	2.6±0.24 p<0.01
2 day (score)	1	4.8±0.2	3±0.55	2.4±0.24 p<0.05
3 day (score)	1	5.2±0.2	2.6±0.6	1.4±0.24 p<0.01
4 day (score)	1	4	2±0.55	1 p<0.01
5 day (score)	1	3.8±0.2	2.2±0.73	1 p<0.01
6 day (score)	1	3±0.32	2±0.55	1 p<0.01
7 day (score)	1	2.2±0.22	1.6±0.67	1 p< 0.05
Open-field				
Crossing (piece)	117.4±2.77	75.8±6.09	98.4±11.45	90±15.99
Grooming (piece)	33±3.61	19.8±1.39	28.6±2.5	30.6±4.37 p< 0.05
Rearing (piece)	1.4±0.93	1.2±0.97	0.6±0.24	1.2±0.73
Elevated plus-maze				
Head dips in to open arm (piece)	6±1.58	10.4±0.51	7±1.13 p<0.05	6.6±1.21 p< 0.05
Enter in to open arm piece)	4.4±1.21	2.6±0.87	3.6±0.26	3.2±0.66
Time in open arm (sec)	46.8±10.17	1.6±0.98	78.8±2.11 p<0.01	68±13.67 p<0.01
Time in closed arm (sec)	253.2±10.17	298.4±0.98	221±2.11 p<0.01	232.2±13.67 p<0.01
Grooming in closed arm	17.6±0.98	12.6±0.51	12.2±1.18	15.4±2.16

Table 4. Effect of 100 μg/rat L-2286 i.c.v. immediately and 30 min post-injury in beam-balance, open-field and elevated plus-maze tests. Data expressed as mean number ± SEM ($p < 0.05$ and $p < 0.01$ compared to vehicle-treated group)

2.3. DISCUSSION AND CONCLUSION

Based on former observations with Marmarou's impact acceleration head injury model, this model results in the impairment of both motor and cognitive functions. In our experiments, to reveal the effectivness of L-2286, we used special immunohistochemical markers of DAI, which reflect NFC and impaired axoplasmatic transport. Based on the results of the two markers, our results indicated that the extent of axonal damage was significantly reduced both in CSpT, which represents the tract of motor functions, and in MLF, which carries sensoric information. The differences in APP and RMO-14 IP axon density reduction confirms the observation, that axonal damage is a heterogenous phenomena, it does not means that the same pathological procedures will be activated in each neuronal fiber.

Based on the experiments apraising of functional outcome, we established, that in this animal model of DAI, -which primarily affects the motor system -, post-treatment with PARP inhibitor was able of improving impaired motor performance, and of reducing the level of anxiety caused by traumatic brain injury. Namely, it improved the functional outcome. Although the present experiments not provide an answer, whether the positive effect of PARP-inhibition is caused by only of the inhibition of DAI, or in Marmarou's head injury model it may have played a role in deflecting diffuse neuronal damage.

3. THE FATE OF "DARK" NEURONS PRODUCED BY TRANSIENT FOCAL CEREBRAL ISCHEMIA IN A NON-NECROTIC AND NON-EXCITOTOXIC ENVIRONMENT: NEUROBIOLOGICAL ASPECTS

Four morphological subtypes of "dark" neurons are currently known: the Huntington type (observed in a mouse model of experimental Huntington disease), the artefactual type (produced by unintentional *post mortem* mechanical injuries of various kinds), the reversible type (early stages of hypoglycemic, epileptic or ischemic injury) and the irreversible type (late stages of hypoglycemic, epileptic or ischemic injury). During the past few years, some researchers have published novel ideas of general neurobiological characters of common nature, common mechanism of formation and common mode of death of the "dark" neurons produced in a visibly normal environment by a head injury, an electric shock or mild hypoglycemia. We can conclude that "dark" neurons have a cellbiological type; the above mentioned four morphological types are the "forced consequences" of their pathological environment.

In this experiment we followed up in time the morphological changes of "dark" neurons -produced by a one-hour intraluminal filament occlusion technique of arteria cerebri media.

3.1. MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200-220g were housed in cages with free access to food and water in the animal facility of the Department of Neurosurgery in Pécs.

Rat transient focal cerebral ischemic model

Animals were anesthetized with a mixture of 2.5% isoflurane, 30% oxygen and 67.5% nitrous oxide during the experiments. The right arteria cerebri media was occluded for one hour by the injury technique of Koizumi et al. The region of the right carotid arteries was exposed, and the external carotid artery, the common carotid artery and its pterygopalatine branch were ligated during the surgery. A 4-0 nylon surgical thread with a flame-rounded tip was inserted into the origin of the middle cerebral artery through an incision in the external carotid artery and kept there for 1 h. Finally, the filament was pulled out and the circulation was re-established. Immediately thereafter, a few rats were perfused transcardially with 500 ml of a glutaraldehyde fixative. The other animals were assigned different survival times. At these time-points, rats were i.p. administered with a 1:1 mixture of 25 mg/ml thiopental and 5 mg/ml diazepam in 2ml/bodyweight before perfusion fixation. In the case of glutaraldehyde fixation, the survival times were the following: 1 h, 4 h, 1 day, 2 days and 1 week, formaldehyde fixation survival times were: 1 h, 1 day and 2 days. Sham-injured animals underwent the same operation procedures, but the filament was removed promptly after its insertion.

Tissue processing

Following transcardial perfusion, brains were removed from the skull after a 1-day delay. Two-thirds of the brains were fixed with glutaraldehyde, and then 150-μm vibratome sections were cut coronally from the caudal end. Every fifth vibratome section was stained by silver technique, which is specific and reproducible for "dark" neurons.

For electron microscopy, 2×2 mm^2 areas of the caudate putamen and the temporal cortex were cut from several remaining vibratome sections, these areas being adjacent to those displaying silver-stained neurons. Ultrastructural investigations were carried out with a Jeol JEM 1200EX transmission electron microscope.

The caudal two-thirds of the brains were fixed with formaldehyde, and then were embedded in paraffin and cut at 10 μm. Every tenth section was stained with 0.1% cresyl violet, or with 1% acid fuchsin. For the demonstration of apoptosis, formaldehyde-fixed sections adjacent to those displaying damaged neurons according to the above staining methods were stained with an *in situ* cell death detection kit (TUNEL), with strict adherence to the instructions featured in the kit manual, and examined under a light microscope.

3.2. RESULTS

Light microscopic observations

In the rats sacrificed immediately after removing of the surgical thread, the silver staining method homogeneously stained the soma-dendrite domain of a few stellate neurons in the right caudate putamen, and a few pyramidal neurons in the right temporal cortex.In the rats that survived for 1 h or longer, the damaged tissue areas (necrotic or excitotoxic) could be recognized on native, unstained tissue sections by their substantially increased phase contrast. In the rats with 1 day survival in those areas of the temporal cortex, contained numerous swollen dendritic and astrocytic processes and many normal-looking neurons, oligodendrocytes, pericytes and endothelial cells, in addition to several "dark" neurons. After 1 h, 4 h and 1 day survival, the somata and dendrites of injured neurons were outlined by mitochondrion-sized silver grains, which are the characteristic features of the recovering "dark" neurons. In the necrotic areas after survival for 1 h, and in the excitotoxic areas of the temporal cortex after 1 and 4 h survival we observed many homogenously silver-stained neurons which individual somata or dendrites could hardly be distinguished.

After 1 or 2 days of survival, the somata of a few stellate neurons and pyramidal neurons remained homogeneously silver-stained, while their dendrites were outlined by silver grains, most of which were much larger than mitochondria, a characteristic feature of dying "dark" neurons.After 1 day of survival, the peripheries (penumbra zones) of injured areas were overcrowded with silver-stained neuronal elements. In those animals, silver staining revealed the characteristic signs of both recovering and dying "dark" neurons, especially in the peripheral zones of excitotoxic areas. In the rats that survived for 6 days, only a few clusters of phagocytotic cells in a visibly normal environment were indicative of dead "dark" neurons.Toluidine blue homogeneously stained the nucleus, cytoplasm and main dendrites of the freshly produced "dark" neurons both in the temporal cortex and in the caudate putamen. In animals that survived for 2 days, the toluidine blue-stained neurons appeared to undergo fragmentation; while after 6 days of survival some fagocytotic cells were observable proving the presence of dying "dark" neurons. TUNEL-positive cell nuclei were present mainly in the penumbra zone of the necrotic foci, mostly in the rats that survived for 1 day.

Electronmicroscopic observations

In the rats sacrificed immediately after the removal of the surgical thread, a few neurons displayed a dramatic compaction. In those neurons distances between ultrastructural elements were dramatically shrunken, the ER cisternae were contracted, while those of the Golgi cisternae were dilated, the mitochondria remained unchanged. The nuclei of such neurons contained several small chromatin clumps. After 1 h survival time, there were many dramatically compacted neurons. Unlike those in the immediately sacrificed rats, these displayed sequestered ribosomes and several slightly swollen mitochondria. Close to those tissue areas with increased phase contrast at 1 h and 4 h of reperfusion, the ER cisternae in several compacted neurons had virtually regained normal width.

In the rats that survived for 1 or 2 days, but not for 6 days, several normal-looking neuronal somata and dendrites contained mitochondrion-sized membranous whorls. A few of them appeared to be just leaving the soma or a dendrite of a neuron. In the same tissue areas a few "dark" neurons became even more compact, so that individual ultrastructural elements could not be distinguished within them, and exhibited many variously sized membrane-bound protrusions. Slightly swollen astrocytic processes that contained numerous glycogen particles surrounded these neurons.

In the 2-day rats, several of these protrusions had become separated into membrane-bound, compact and homogeneous fragments. In the rats that survived for 6 days, such neurons and neuronal fragments were partly or completely engulfed by microglial cells, or by astrocytes.

In the necrotic or excitotoxic tissue areas of the rats that survived for 1 day, a few "dark" neurons were even more compact and more electron dense, while others became swollen, and in part or fully disintegrated. Such neurons were surrounded by watery neuronal dendrites or extremely swollen astrocytic processes that did not contain glycogen particles.

In the rats that survived for 2 or 6 days, "dark" neurons underwent necrotic-like disintegration. In those rats that survived for 1 day, several neurons with a compacted ultrastructure in the penumbra zone of the necrotic area contained a few large, rounded (apoptotic) chromatin clumps. In the rats that survived for 2 days or longer, several neurons had undergone a necrotic-like disintegration. Others had fallen into membrane-bound, compact and homogenous fragments that were surrounded by glycogen-containing astrocytic processes. Those were partialy or completely engulfed by microglial cells or by astrocytes.

3.3. DISCUSSION AND CONCLUSION

By definition, ischemia means the complete arrest of blood circulation. However, in our experiment this was only partly true, because the blood flow was reduced in a different degree in the caudate putamen's necrotic area, the temporal cortex citotoxic area, and in a visibly intact neighbouring areas. For this reason, the pathobiochemical cascade that initiates ultrastructural compaction might be quite different in these tissue areas. Although, the essence of the morphological events was the same in each freshly produced "dark" neuron both in stellate neurons in the caudate putamen and pyramidal neurons in the temporal cortex. Furthermore, they were in aggreement of the morphological pictures of "dark" neurons caused by other (mechanical, electric, hypoglicemic) noxae.

From these observations we can conclude, that the formation of "dark" neurons has an initiation and an execution phase. The mechanism of initiation can be varied, while the mechanism of executive phase is independent on the phenotype, chemotype and function of the affected neurons. This fact strenghtens our former hypothesis pertaining to the formation of "dark" neurons.

One half of the “dark” neurons that are caused by traumatic brain injury, electric shock and hypoglicemia will regenerate. As the first event, the ER cisternae regain their original width. Thereafter, the degree of ultrastructural compaction gradually decreases. During this process, mitochondrion-sized membranous whorls appear in the somata and the main dendrites of the affected neurons and after 1 or 2 days they leave the neurons. In our case we observed the same morphological changes by recovering “dark” neurons.

The other half of the “dark” neurons caused by traumatic brain injury, electric shock and hypoglicemia will die. As impact of the above mentioned noxae, the dying portion of compacted neurons in non-necrotic, non-excitotoxic, respectively non-contusion brain areas will be removed in similar manner to apoptotic cells, which was found in our case too. We may draw the conclusion that “dark” neurons are not dying via the necrotic pathway, it was once thought that “dark” neurons in necrotic, cytotoxic, and contusioned brain areas are dying in a similar manner to necrotic neurons. In the case of the above mentioned physical noxae ultrastructural compaction is immediate, making the apoptotic cell death pathway unimaginable. Therefore, “dark” neurons must have their own dying mechanism.

In our case apoptotic cells in necrotic environment were removed in a similar manner to necrotic cells. Only residual chromatin lumps referred to their apoptotic origin. Namely, the necrotic environment forced a necrotic removing mechanism to neurons dying by apoptotic cell death pathway. By analogy, we assume that the necrotic environment pushes a necrotic removing mechanism to dying “dark” neurons.

III. SUMMARY OF NOVEL FINDINGS

1. In our experiments with a moderate CFP model, we found that treatment with 100 μg PACAP i.c.v. 30 minutes post-injury significantly reduced the density of both APP and RMO-14 IP axons. PACAP has a neuroprotective effect in both forms of TAI: in AS/B formation as well as in NFC.

The neuroprotective effect of PACAP was not significant in MLF. This can be explained by the fact that in this tract, the density of intact axons, their diameter and course is different than in the CSpT, where the neuroprotective effect proved to be significant.

Our results supported former observations from the impact acceleration model of TAI, that PACAP efficiently reduces the number of impaired axons in cases of both impaired intraaxonal transport and NFC caused by traumatic brain injury.

2. In our experiments with Marmarou’s impact acceleration model we found that immediate or 30 minutes post-injury i.c.v. treatment with 100 μg/rat of L-2286 (a newly developed PARP inhibitor) significantly reduced the density of both APP and RMO-14 IP axons in both CSpT and MLF. Therefore, L-2286 has a neuroprotective effect in both forms of TAI: in AS/B formation as well as in NFC.

Treatment with 100 μg/rat L-2286 i.c.v. had a different reducing effect to the density of APP and RMO-14 IP axons. This fact suggests that these axonal damages are heterogeneous phenomenas, and in different neuronal fibers they will be „activated” with different chance.

The i.c.v. treatment with 100 μg/rat L-2286 also proved to be neuroptotective in behavioral tests. Namely, in the beam-balance test it significantly improved impaired motor functions, furthermore significantly reduced the raised anxiety level in the elevated plus-maze test.

3. We made new observations about the formation and morphological aspects of “dark” neurons in transient focal cerebral ischemia model.

The basic morphological features of every freshly produced “dark” neuron was the same in all examined areas (central, and peripheral areas of caudate putamen, in addition to cytotoxic areas of the temporal cortex, and its visibly intact neighbouring areas), despite the different blood flow rates. These features were in agreement with the morphological pictures of “dark” neurons caused by other injury-causing (mechanical, electric, hypoglicemic noxae) impacts.

From these observations, we conclude, that the formation of “dark” neurons has an initiation and an execution phase. The mechanism of inititation can be varied, while the mechanism of executing phase is independent from the type of initiating noxae, as well as the phenotype, chemotype and function of the affected neurons. This fact supports our former hypothesis pertaining to the formation of “dark” neurons. We assume that the necrotic environment pushes the dying “dark” neurons to a necrotic removing mechanism.

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1. J Pál, L. Kellényi, **E. Kövesdi**, J. Lückl, E. Ezer, F. Gallyas, A. Büki, T. Dóczi: Rodent model of multiparametric intracranial pressure monitoring. MIT 17th Congress, 3rd Pannonian Symposium on CNS Injury, Pécs, Hungary, 2005

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