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Unexpected morphological changes within hippocampal structures in a photochemical ring model of cerebral ischaemia

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Abstract

A photochemical ring model of ischaemia was introduced in the middle of the nineteen eighties. Irradiation by a laser or arc lamp followed by intravenous injection of rose bengal resulted in thrombosis of pial and superficial cortical vessels. This ring model imitated focal ischaemic damage in humans.

In our experiment twenty-seven Wistar rats of both sexes weighing 250-300 grams were examined. A photochemical ring model based on irradiation of the area of parietal bone 4 mm posteriorly to the bregma and 4 mm laterally from the sagittal suture was applied. A ring-shaped light beam with a wavelength of 510-540 nm with 5 mm diameter was generated by a high pressure discharge lamp at a power of 400 W. Two groups of rats treated and untreated with MK-801 and two rings of the thickness of 0.35 mm and 0.5 mm were used in the experiment. Morphological examination was performed in animals sacrificed 1 and 4 days after the irradiation. On formalin-fixed and paraffin-embedded slices HE staining method and immunoreaction with antibodies to ubiquitin were applied.

Our material confirmed well known information about the dynamics of infarct breakdown, ischaemic-induced angiogenesis, glial reaction and other typical changes described previously in handbooks and numerous papers. In the experiment, morphological changes were more intensive after the irradiation by 0.5 mm than 0.35 mm irradiating rings and 4 days than one day after the irradiation.

A surprising finding observed in some of the examined animals was more intensive neuronal damage after treatment with MK-801. Another unpredicted discovery was intensive morphological alterations found in CA4 and CA3 hippocampal sectors. Moreover, these alterations were not limited to the damaged hemisphere, but were also observed contralaterally. In some of the rats, ischaemic and necrotic cells were additionally found within both parasagittal areas. We connect this atypical localization of the ischaemic changes with dispersion of light emitted by the used lamp. Dispersed light also leads to thrombotic occlusion of the meningeal arteries in the parasagittal area. Among these arteries, thrombosis in pericallosal and penetrating arteries was present.

Our experiment demonstrated that if a non-laser lamp is used, brain areas distant from the necrotic ring must be carefully investigated.

Key words: *fotochemical ring model, cerebral ischemia, MK-801, hippocampal areas.*

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Introduction

The photochemical model of brain ischaemia using a xenon lamp or argon laser-induced photothrombosis was introduced in the middle of the nineteen eighties by Watson et al. (1985) [20]. In these experiments, intravenous injection of rose bengal followed by irradiation with green light resulted in thrombosis of pial and superficial cortical vessels. Two years later this model was modified by Wester et al. (1995) [21], who introduced laser-induced photothrombotic cortical lesions in the shape of a ring with 5 mm external diameter and a thickness of 0.5 mm. Further modifications of this method introduced reducing ring thickness to 0.35 mm. This ring imitated focal ischaemic damage in humans with penumbra located within the ring. In this model the intensity of an irradiating beam influences the degree of ischaemic damage [8]. In the beginning, an evident decrease of cerebral blood flow (CBF) within the cerebral cortex and ischaemia is observed, then, between 24 and 48 hours, CBF gradually increases and finally normalizes after seven days [5]. In the photochemical model of cerebral ischaemia widespread alterations were found [3]. Estimation of penumbra evolution [21] indicated participation of pro- and anti-apoptotic proteins [9]. Very interesting correlation between histopathological, biochemical and MRI investigations was carried out by Hilger et al. [6]. In the experiment, MRI imaging turned out to be more useful in estimation of penumbra in animals than in humans. Within the centre of the ring ischaemic lesion, cerebral blood flow and protein synthesis exhibit reversible alterations [11].

Various pathogenic factors acting in ischaemic lesions have been investigated. In the damaged hemisphere, increased expression of NMDA receptors was seen 14 days after the irradiation, whereas in the contralateral hemisphere it appeared much earlier, after 4 hours [5,16]. Irradiation also produced alterations in neuronal network excitability in CA1 and CA3 hippocampal areas [3]. Moreover, it was found that intensity of an irradiating beam influenced the degree of ischaemic damage [8].

The photochemical model of ischaemic lesion using a ring-shaped laser-irradiation beam is very useful in estimation of penumbra and an influence of neuroprotective drugs on this "zone at risk". In our experiment we tested two lamp-generated rings with irradiating beams of 0.35 mm and of 0.5 mm. In some of the experimental animals the influence of MK-801 was estimated. MK-801 is an NMDA receptor antagonist inducing *in vivo* neuroprotection in several models of stroke [2] and preventing acute [23] and chronic [22] homocysteine neurotoxicity. In experiments of glutamate neurotoxicity in vitro, a weak neuroprotective effect of 1-methylnicotinamide was also observed. But this effect is not connected with direct inhibition of NMDA receptors [18].

The goals of our experiment were the following:

1. Appreciation of morphological changes after irradiation.
2. Comparison of morphological pictures due to irradiation rings of different size.
3. Assessment of MK-801 effect.

Material and Methods

Twenty-seven Wistar rats of both sexes weighing between 250 and 300 grams were anaesthetized with a 3% solution of chloral hydrate in 0.9% NaCl at a dose of 325 mg/kg peritoneally. During general anaesthesia, the skin on their heads and shank was cut in order to reveal the parietal bone and saphenous vein. A solution of the photosensitizing dye erythrosine B (12.96 mg/ml in 0.9% NaCl) prepared in advanced and kept in a covered container was injected intravenously at a dose of 35 mg/kg from a covered syringe. In order to avoid a rapid decrease of blood pressure, erythrosine was slowly injected for about two minutes.

Animals prepared in such a way were mounted on a stereotactic frame. Annular damage of cortex of 5 mm in diameter was achieved by a photochemical method. This method is based on irradiation of the parietal bone area 4 mm posteriorly to the bregma and 4 mm laterally from the sagittal suture. A green light with wavelength of 510-540 nm, generated by a high pressure discharge lamp at a power of 400 W, in which the discharge was made in superheated vapour of mercury and thallium halide, was applied to this area. The structure of the condenser set, thanks to the centrally placed diaphragm, allows the lamp light to be directed only on the circumference of the irradiated area, causing annular damage of the cortex with external diameter of 5 mm. It also allows regulation of the thickness of the irradiated ring ranging from 0.5 mm to 35 mm with a constant time of irradiation which was 5 minutes.

Treated and untreated MK-801 rats used in the experiment were divided into groups according to the schematic diagram (Table I).

MK-801 (Sigma) in the 0.5 solution in 0.9% NaCl was applied peritoneally at doses of 5 mg/kg one hour before irradiation.

After the allocated time of survival of the rats in the experiment, the animals were anaesthetized again with a solution of chloral hydrate and transcardially perfused with a solution of 4% formalin. The dissected brains were postfixed in perfuse liquid, dehydrated in alcohol and embedded in paraffin. The microtome sections of 8 um thickness comprising the irradiated area were stained with cresyl violet, haematoxylin-eosin and ubiquitin according to earlier described procedures [17]. Morphological changes caused by irradiation were analyzed under a light microscope.

Results

Since morphological changes in the ring photochemical model of cerebral ischaemia have been relatively well characterized, we limit the report to only new morphological findings. Our experiment revealed the presence of very evident morphological changes within CA4 and CA3 areas of the hippocampus and in the vicinity of these structures and we would like to focus only on these atypical changes.

Irradiating beam – 0.35 mm

1 day; rats without treatment

Hippocampal tissue indicated the severe destructive influence of ischaemia. In the CA4 segment many dark and ischaemic neurons were found, while single necrotic cells were visible only in CA3 and CA4 (Table II) (Fig. 1).

1 day; rats after treatment

In spite of the treatment, inconsiderable numerous ischaemic and dark neurons were found in the CA3 and CA4 regions and even in CA1. Numerous necrotic cells were observed only in two cases (Figs. 2, 3) while in other cases they were only single. Necrotic cells were seen also in the ipsilateral parasagittal area (Table II) (Fig. 4).

Table I. Material

Time of irradiation	Ring diameter	Ring thickness	Survival time	Untreated MK-801	Treated MK-801	Number of animals
5 min	5 mm	0.35 mm	1 days	3	4	7
5 min	5 mm	0.35 mm	4 days	4	3	7
5 min	5 mm	0.5 mm	1 days	3	3	6
5 min	5 mm	0.5 mm	4 days	3	4	7

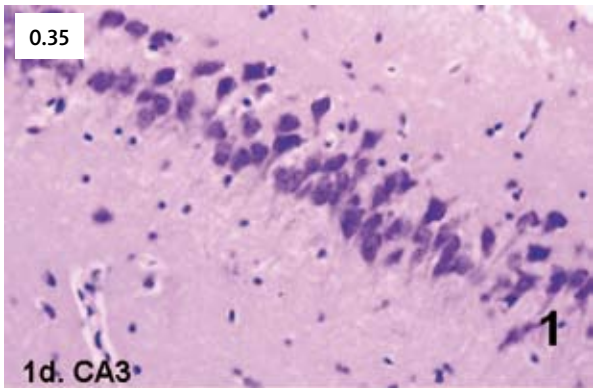
Table II. Irradiating beam 0.35 mm, 1 day Histopathological changes within hippocampus and other brain regions

1 day		Ischemic or dark neurons			Necrotic neurons						
	Nr	CA1	CA3	CA4	CA1	CA3	CA4	Parasag-gital I-L	Parasag-gital C-L	Dentate gyrus I-L	Dentate gyrus C-L
without treatment	56/03			++			+_				
	57/03		+			+_					
	58/03			++							
MK-801 treatment	77/03	++	++	+	+_	++	+++	+		+	
	78/03		++	+++			+_				
	79/03		+++	+++			+_	++			
	76/03		++	++		+	+_	+	+		

I-L – ipsilateral; C-L- contralateral; single +_; non numerous +; numerous ++; very numerous +++

4 days; rats without treatment

Pathological changes were observed both in the damaged and contralateral brain hemisphere (Table III) (Fig. 5). A moderate number of ischaemic and dark neurons were noted only in the CA4 sector. Necrotic neurons were more numerous, although in 2/4 cases they were visible in the hippocampus only sporadically. In cases with a more evident reaction, necrotic neurons predominated in the CA4 segment (Table III) (Fig. 5). Necrotic cells were also found bilaterally in the parasagittal region and in the dentate gyrus. They were immunoreactive to ubiquitin (Fig. 6).



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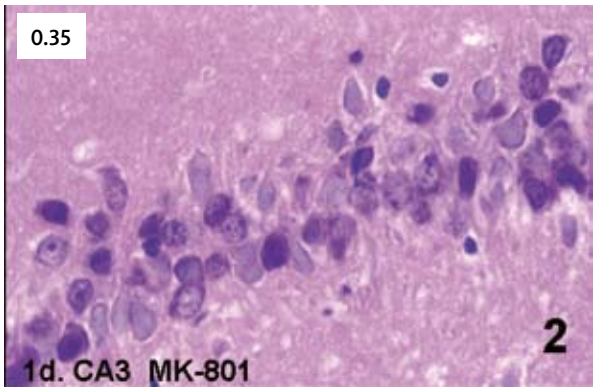

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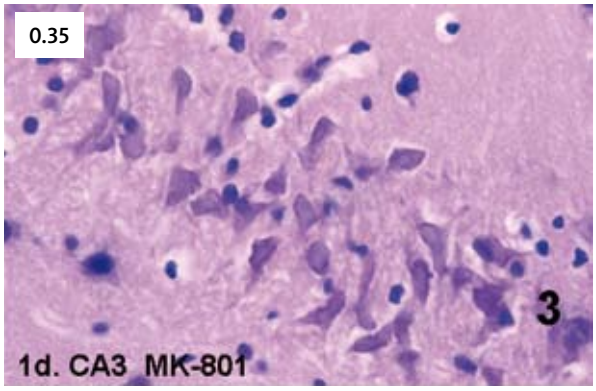

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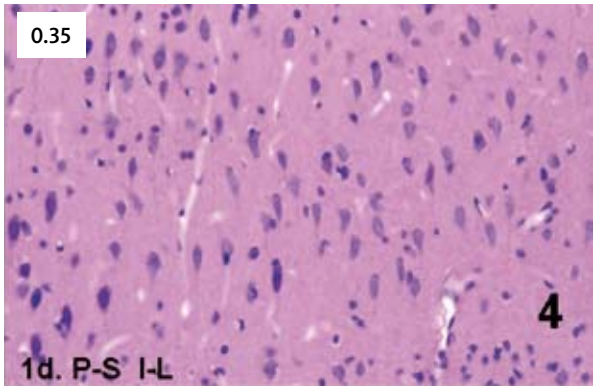

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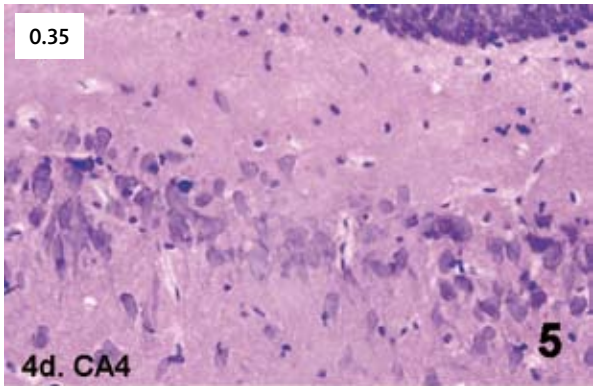

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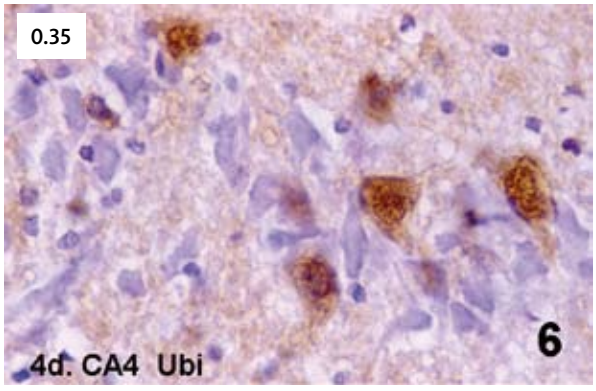


Fig. 6.

Table III. Irradiating beam 0.35 mm, 4 days Histopatological changes within hippocampus and other brain regions

4 day		Ischemic or dark neurons			Necrotic neurons						
	Nr	CA1	CA3	CA4	CA1	CA3	CA4	Parasag-gital I-L	Parasag-gital C-L	Dentate gyrus I-L	Dentate gyrus C-L
without treatment	60/03				+	++	+++			+	+
	61/03			+			+_				
	62/03			+_			+_				
	63/03			+_	+_		++	+	+		
MK-801 treatment	73/03	+_	+	+	+_	+	+_	+	+		
	74/03		+	++		+_	+_				
	75/03			+			+_				

I-L – ipsilateral; C-L – contralateral; single +_; non numerous +; numerous ++; very numerous +++.

4 days, after treatment by MK-801

Ischaemic and dark neurons, particularly in the CA4 area, and sporadically necrotic cells within CA3 and CA4 were found. Parasagittal necrotic cells were present bilaterally in 1/3 cases (Table III).

Irradiating beam – 0.5 mm

Histopathological changes were more intensive and widespread than after irradiation with a beam of 0.35 mm.

1 day, rats without treatment

Many dark and ischaemic neurons within CA3, CA4 and even CA1 segments were present. Single necrotic neurons were found in CA1 and CA3 while in CA4 they were numerous (Table IV). All kinds of neuronal changes were observed bilaterally in parasagittal areas (Table IV) (Figs. 7, 8). Ischaemic and dark neurons were additionally seen in the frontal cortex, while necrotic cells were noted in the subiculum and both dentate gyruses.

1 day, rats after treatment with MK-801

Necrotic cells were observed bilaterally in CA4, but were more abundant in the damaged hemisphere (Table IV) (Figs. 9, 10). Comparison between animals treated and untreated with MK-801 revealed that only in the CA3 segment was neuronal damage less severe after the drug treatment than without it.

4 days, rats without treatment

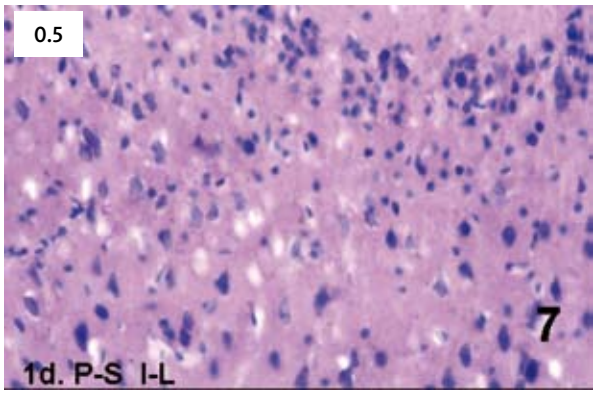
Histopathological changes were moderately severe and widespread. In the ipsilateral hippocampus ischaemic and dark neurons prevailed in the morphological picture. Both kinds of cell changes were also observed in other brain regions: in the parasagittal area (Table V) (Figs. 11, 12) and in the contralateral CA3 and CA4 segments. Necrotic cells were not numerous and a slight loss of cells was observed in CA4. Relatively numerous necrotic cells were noted in both parasagittal areas.

4 days, rats after treatment by MK-801

A therapeutic effect of MK-801 was not observed and morphological pictures were similar in treated and untreated animals (Table V). In the CA4 segment neuronal damage seemed to be even deeper and evident loss of CA4 cells was found (Fig. 13). In some animals thrombosis of the pericallosal arteries was visible (Fig. 14). Vessel thrombosis was also observed in perforating ramifications of the pericallosal arteries (Fig. 15).

Discussion

Evaluation of our experimental material from the rat photochemical ring model of cerebral ischaemia provided information concerning: dynamics of ischaemic cell injury development, differences in histopathological ischaemic changes triggered by two irradiating rings, morphological picture of penumbra



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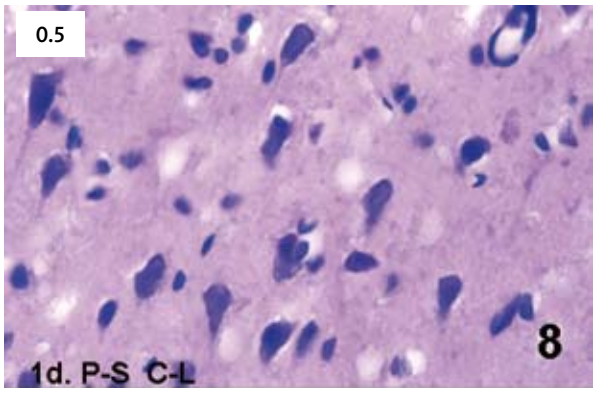

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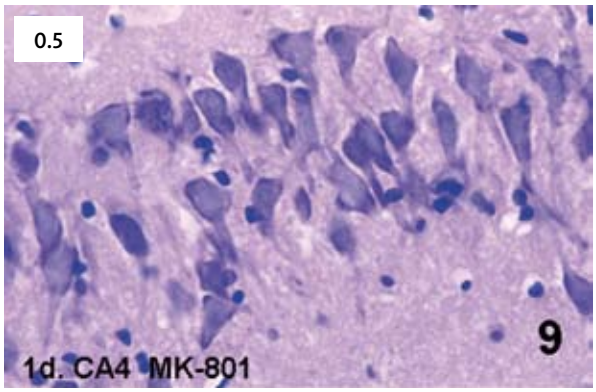

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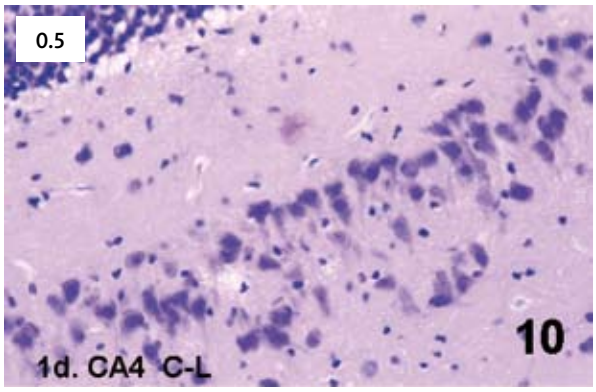

Fig. 10.

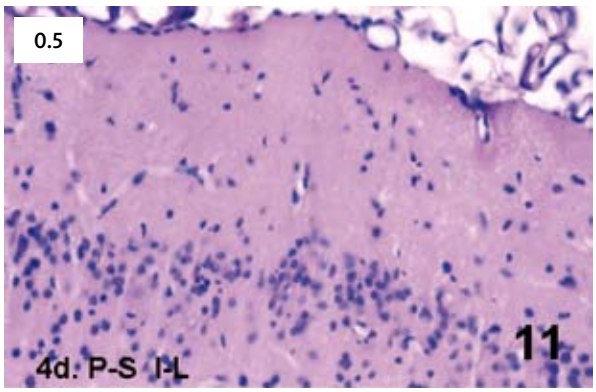

Fig. 11.

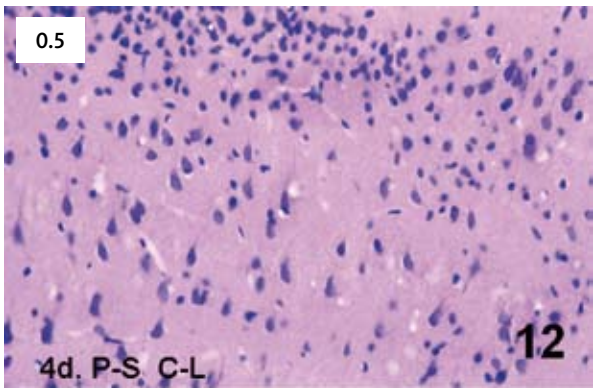

Fig. 12.

after irradiating, atypical distant damage of hippocampus and therapeutic effect of MK-801.

In the majority of the experimental investigations on brain ischaemia, particularly in global brain ischaemia, the CA1 hippocampal area was more vulnerable to ischaemic injury than other segments. In the photochemical ring model alterations in hippocampal structures were observed very rarely. Contrary to previous investigations, in our experiment hippocampus damage was evident. Surprisingly, not CA1 but CA3 and, especially, CA4 turned out to be severely injured. This finding, as well as other results of our experiment, raises a few

Table IV. Irradiating beam 0.5 mm, 1 day Histopatological changes within hippocampus and other regions

1 day		Ischemic or dark neurons				Necrotic neurons							
	Nr	CA1	CA3	CA4	Other regions	CA1	CA3	CA4	Para-saggital I-L	Para-saggital C-L	Dentate gyrus I-L	Dentate gyrus C-L	Other
without treatment	1/03	+_	+_	++	PS, F ++			+	++	++	+_	+	F
	2/07	++	++	++	PS +++		+_	+	++	++	+	+	
	3/07	+	+	++	PS, F ++	+_		+++		++	+	+	Sub
MK-801 treatment	64/03	+		+++				++	++	+			
	65/03		+	++	CA4 C-L	+_	+_	+++	++	+			CA3 CA4 C-L
	67/03		+	++				+_	+_				CA4 C-L

PS – parasaggital area; I-L – ipsilateral; C-L – contralateral; F – frontal cortex; Sub – subiculum; single +_; non numerous +; numerous ++; very numerous +++.

Table V. Irradiating beam 0.5 mm, 4 days Histopatological changes within hippocampus and other brain regions

4 day		Ischemic or dark neurons				Necrotic neurons							
	Nr	CA1	CA3	CA4	Other regions	CA1	CA3	CA4	Para-saggital I-L	Para-saggital C-L	Dentate gyrus I-L	Dentate gyrus C-L	Other
without treatment	4/07	+	+	+	I-L, PS, ++			+_		+_			
	5/07	++	++	++	C-L, CA3, CA4, ++								
	6/07	++	++	++	C-L, CA3, CA4, ++ C-l, I-L, PS, ++	+_	+_	+_	++	+			
MK-801 treatment	68/03			+				++	+_				
	72/03	++	+	+		+		+_	+				CA4 +_
	69/03	+_	+	++				+_	+				
	70/03	+_	++	+++	C-L, Hip +_	+	+_	+++	+				F

C-L – contralateral; I-L – ipsilateral; F – frontal cortex; PS – parasagital area; Sub. – subiculum; Hip – hippocampus; single +_; non numerous +; numerous ++; very numerous +++

problems requiring more detailed discussion. We limit our considerations to the following issues:

1. Morphological changes caused by the irradiating beam.
2. Comparison of morphological pictures elicited by the 0.35 mm and 0.5 mm irradiating beams.
3. Influence of MK-801 treatment on morphological changes.
4. Possible causes of hippocampal and parasagittal damage.

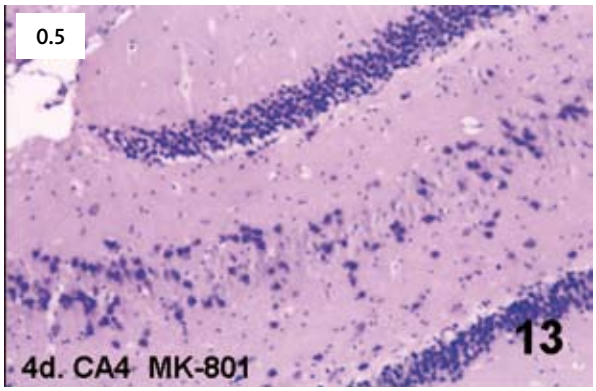

Fig. 13.

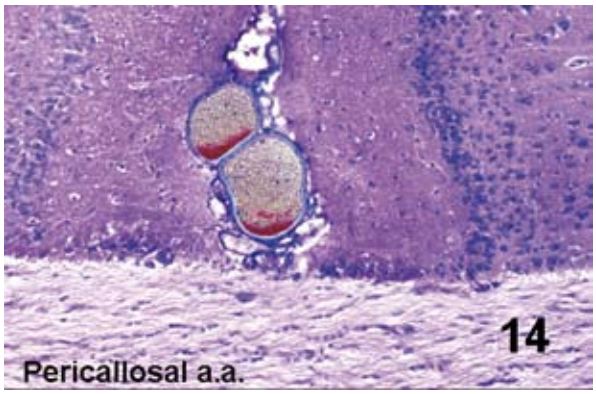

Fig. 14.

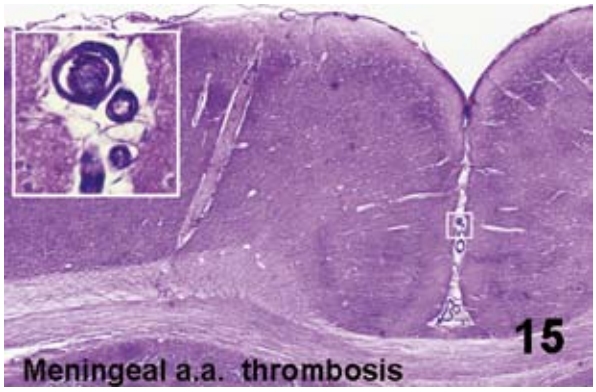

Fig. 15.

Point 1. Morphological changes caused by the irradiating beam

On the 4th day after the irradiation, ischaemic and necrotic changes were more intensive than on the first day. Morphological manifestations of ischaemia and dynamics of their development were the same as in the ischaemic model due to vessel occlusion. These changes are well known and there are many publications concerning this issue in the literature and handbooks.

Point 2. Comparison of morphological pictures elicited by the 0.35 mm and 0.5 mm irradiating beams

Similarly to other observations [10], irradiation by the 0.5 mm ring caused more intensive and extensive destructive changes than the 0.35 mm ring.

Point 3. Influence of MK-801 treatment on morphological changes

In our material on the first day after MK-801 treatment, surprisingly more neurons were damaged than in animals without the treatment. Neuronal injury was observed both in the irradiated and contralateral hemisphere. The neuroprotective potential of MK-801 in the rat model of focal brain ischaemia has been described in several studies (e.g. 2). There are a few possible explanations of this finding. Experimental [15,19] and human [4] studies revealed that sometimes MK-801 can evoke a neurotoxic effect. It is possible that increased damage to neurons observed in the treated animals may be a manifestation of such toxicity. Another cause of this finding may be associated with difficulties in appreciation and interpretation of the results of our experiment. In 1998 Hossmann [7] proposed five strong criteria which must be fulfilled to obtain reliability of results of experimental studies. But apart from these criteria, some additional problems await morphologists. First of all, in animals, even minimal displacement of the irradiating lamp toward the bregma, in both vertical and horizontal plane, leads to impossibility to obtain identical hemispheric cross-section. Moreover, comparison of brain slices originating from different animals affords great difficulties due to individual differences in brain size. Also, with every consecutive tissue slice the distance from the ischaemic lesion increases. The above factors influence estimation of ischaemic lesion size and, in consequence, extensiveness and intensity of brain oedema, activity of breakdown process, intensity of angiogenesis and glial

reactivity. Morphometric examination may help in estimation of grade of tissue destruction, but results are reliable only in some of the brain structures (CA1 hippocampal area, lamina of Purkinje cells in cerebellum, anterior horns of the spinal cord and motor nuclei of the cranial nerves). Taking into account all the limitations mentioned above, we conclude that detailed comparison of morphological changes in various animals is impossible or laden with too great mistakes. Difficulties in comparison of morphological changes in different rats and various sensitivity of individual animal to MK-801 result, in turn, in difficulties in judging the drug's therapeutic effects.

Point 4. Possible causes of hippocampal and parasagittal damage

In all experimental animals, apart from typical ischaemic and necrotic lesions, some unexpected changes were also found. In the CA3 and CA4 hippocampal sectors and in the parasagittal area, dark ischaemic and necrotic neurons were present. Although damage to CA3 and CA4 has been described in global brain ischaemia [1,13,14] and in cerebral hypoxic injury [12], we did not find any data in the literature referring to the presence of such morphological changes in the photochemical ring model of cerebral ischaemia.

The question arises, what was the cause of the damage to these regions. In various long-term experiments, evident injury of the corpus callosum was demonstrated, which might explain the presence of damaged neurons in structures distant from the irradiating areas. But in our material 4 days after the irradiation, the corpus callosum was not damaged. The most probable explanation of the bilateral localization of dark neurons in the hippocampus and parasagittal area seems to be thrombotic occlusion or significant decrease of cerebral blood flow in ramification of the anterior cerebral artery. One of the main branches of the anterior cerebral artery is the symmetric pericallosal artery running longitudinally to the corpus callosum. Penetrating branches of pericallosal arteries supply the parasagittal cerebral cortex. Occlusion or thrombotic stenosis of the pericallosal arteries or their branches may lead to the development of the morphological changes observed by us. Various depth of penetration of pericallosal arteries ramifications may also explain the presence of neuronal changes in the contralateral CA4 hippocampal area. A morphological study performed in non-perfused animals showed thrombotic occlusion of vessels within the meningeal interhemispheric space. Unfortunately, in animals after perfusion, finding a thrombosis in blood vessels is very difficult. It seems that only a laser lamp can cause ring thrombotic changes in meningeal blood vessels without producing distant changes. A non-laser lamp is a source of dispersed light and the distant morphological changes observed in our material may be a result of this dispersion.

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